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Acetyl Coenzyme A Synthetase Is Acetylated on Multiple Lysine Residues by a Protein Acetyltransferase with a Single Gcn5-Type *N*-Acetyltransferase (GNAT) Domain in *Saccharopolyspora erythraea*

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Reversible lysine acetylation (RLA) is used by cells of all domains of life to modulate protein function. To date, bacterial acetylation/deacetylation systems have been studied in a few bacteria (e.g., *Salmonella enterica*, *Bacillus subtilis*, *Escherichia coli*, *Erwinia amylovora*, *Mycobacterium tuberculosis*, and *Geobacillus kaustophilus*), but little is known about RLA in antibiotic-producing actinomycetes. Here, we identify the Gcn5-like protein acetyltransferase AcuA of *Saccharopolyspora erythraea* (*Sac*AcuA, SACE_5148) as the enzyme responsible for the acetylation of the AMP-forming acetyl coenzyme A synthetase (*Sac*AcsA, SACE_2375). Acetylated *Sac*AcsA was deacetylated by a sirtuin-type NAD^+-dependent consuming deacetylase (*Sac*SrtN, SACE_3798). *In vitro* acetylation/deacetylation of *Sac*AcsA enzyme was studied by Western blotting, and acetylation of lysine residues Lys^{237}, Lys^{380}, Lys^{611}, and Lys^{628} was confirmed by mass spectrometry. In a strain devoid of *Sac*AcuA, none of the above-mentioned Lys residues of *Sac*AcsA was acetylated. To our knowledge, the ability of *Sac*AcuA to acetylate multiple Lys residues is unique among AcuA-type acetyltransferases. Results from site-specific mutagenesis experiments showed that the activity of *Sac*AcsA was controlled by lysine acetylation. Lastly, immunoprecipitation data showed that *in vivo* acetylation of *Sac*AcsA was influenced by glucose and acetate availability. These results suggested that reversible acetylation may also be a conserved regulatory posttranslational modification strategy in antibiotic-producing actinomycetes.

Reversible lysine acetylation (RLA) of proteins is now recognized as a ubiquitous and conserved posttranslational modification in a variety of organisms (1–4). Recent studies have identified over 2,000 acetylated proteins, ranging from transcriptional factors and ribosomal proteins to metabolic enzymes related to glycolysis, gluconeogenesis, the tricarboxylic acid (TCA) cycle, and fatty acid and nitrogen metabolisms. This kind of posttranslational modification (PTM) has emerged as an important metabolic regulatory mechanism in bacteria since the discovery of acetylation of the *Salmonella enterica* acetyl coenzyme A (Ac-CoA) synthetase in 2002 (5). In the last decade, lysine acetylation of proteins has been reported in other microorganisms, including *Escherichia coli*, *Bacillus subtilis*, *Streptomyces lividans*, *Mycobacterium tuberculosis*, *Erwinia amylovora*, *Thermus thermophilus*, and *Geobacillus kaustophilus* (6–9).

RLA has been found to modulate protein synthesis, central metabolism, and detoxification metabolism. Yu et al. identified 85 acetylated proteins in *E. coli*, of which 24 (28%) are involved in protein biosynthesis and 16 (19%) are involved in carbohydrate metabolism (10). Zhang et al. also reported that more than 70% of the 91 acetylated proteins in *E. coli* are metabolic enzymes (53%) and translation regulators (22%) (11). More recently, Wang et al. identified 235 peptides containing acetylated lysines in a total of 191 proteins in *Salmonella enterica* and found that enzymes involved in the central metabolism are extensively acetylated and that their acetylation profiles change in response to different carbon sources, concomitantly with changes in cell growth and metabolic flux (2). These results suggest an extensive role of acetylation in the regulation of intracellular metabolism in response to rapidly changing conditions.

The acetate-scavenging, AMP-forming acetyl-CoA synthetase (Acs; EC 6.2.1.1; acetate:CoA ligase) was the first enzyme reported to be regulated by acetylation in prokaryotes and has been extensively investigated in *S. enterica* (5), *B. subtilis* (12), *E. coli* (13), *Rhodopseudomonas palustris* (14), *M. tuberculosis* (15), *Streptomyces lividans* (6), and *Saccharomyces cerevisiae* (16). AMP-forming acetyl-CoA synthetases belong to the acyl-adenylate-forming superfamily, are ubiquitous enzymes whose activity is central to the metabolism of prokaryotic and eukaryotic cells, and are responsible for the assimilation of acetate. AMP-forming acetyl-CoA synthetases catalyze the reaction acetate + CoA + ATP ↔ acetyl-CoA + AMP + PP_i, for the reversible conversion of acetate to acetyl-CoA (Ac-CoA), providing the cell the two-carbon metabolite used in many anabolic and energy generation processes.

In *E. coli*, it is known that the expression of the *acs* gene is strictly controlled by complex regulatory systems at a transcriptional level (17, 18). Notably, the activity of Acs is also regulated at the posttranslational level by the RLA system. All RLA systems in prokaryotes consist of protein acetyltransferases (referred to as Gcn5-type *N*-acetyltransferases, or GNATs) and protein deacetylases. GNATs are conserved in all domains of life and catalyze the transfer of the acetyl from the Ac-CoA donor to a primary amine of small molecules and proteins. The GNAT protein superfamily contains over 50,804 members (pfam00583), which are involved

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in a wide variety of cellular processes. GNAT enzymes that modulate the acetylation state of histones for facilitating transcription (hyperacetylated histones) or gene silencing (hypoacetylated histones) in eukaryotic cells have been extensively studied. However, so far, only a few GNATs responsible for the acetylation of proteins have been identified in bacteria. In *Salmonella enterica*, the protein acetyltransferase Pat (*Se*Pat) inactivates acetyl-CoA synthetase (*Se*Acs) through lysine acetylation (19). Similar observations have been reported in other Gram-negative bacteria that synthesize *Se*Pat homologues, such as *E. coli* and *Rhodopseudomonas palustris* (14, 20). RLA has also been investigated in Gram-positive bacteria such as *Bacillus subtilis*, whose genome encodes a protein acetyltransferase AcuA (*Bs*AcuA) that modifies the acetyl-CoA synthetase of this bacterium (12).

We are interested in acetylation of protein in high G+C DNA Gram-positive actinomycetes, which are important producers of therapeutic antibiotics. In some pathogenic actinomycetes, e.g., *M. tuberculosis* and *Mycobacterium smegmatis*, two protein acetyltransferases, PatA and Pat (*Mt*PatA and *Ms*Pat, respectively), were found to acetylate acetyl-CoA synthetase and a universal stress protein (15, 21). The *Se*Pat homologue of *Streptomyces lividans* (*Sl*PatA) has protein acetyltransferase activity that modulates the activity of the acetoacetyl-CoA synthetase enzyme of this bacterium (6). Acetyl-CoA synthetase was also found to be regulated *in vivo* by acetylation in *Streptomyces coelicolor*, but the acetyltransferase responsible for this acetylation was not identified (22). The *B. subtilis* AcuA (211 residues) and *Rhodopseudomonas palustris* KatA (*Rp*KatA) GNAT enzymes are much smaller than *Se*Pat-type acetyltransferases since they contain only the GNAT domain. Of relevance to this work is the fact that all previously reported bacterial acetyltransferases acetylate only one lysine residue (in boldface) within the conserved acylation motif PXXXXG**K** found in AMP-forming acyl-CoA synthetases.

At present, our understanding of the physiological role of RLA in high G+C-content Gram-positive bacteria (e.g., *Streptomyces* and *Saccharopolyspora erythraea*) is limited, the function of protein acetyltransferases responsible for acetylation of specific proteins has not been described, and the systematic screening of Pat substrates has not been investigated. Actinomycete genomes generally encode more than 40 protein acetyltransferases (http://pfam.xfam.org/family/PF00583; http://www.ebi.ac.uk/interpro/entry/IPR000182). To understand the role of acetylation in bacteria, it is imperative to identify the functions and biochemical characterization of these enzymes. Further understanding of the RLA regulation of metabolism in actinomycete species is of interest because of the diversity of natural products synthesized by these organisms.

In this study, we identified the Gcn5-like *S. erythraea* protein acetyltransferase (SACE_5148; hereinafter referred to as *Sac*AcuA) and sirtuin-type NAD-dependent deacetylase (SACE_3798; hereinafter referred to as *Sac*SrtN) responsible for acetylation/deacetylation of acetyl-CoA synthetase (SACE_2375; hereinafter referred to as *Sac*AcsA) and found that *Sac*AcuA, unlike its *B. subtilis* homologue, can acetylate multiple lysine sites of its protein substrate. Furthermore, it was also demonstrated that the acetylation status of *Sac*AcsA is modulated by extracellular nutrient availability. These results suggested that reversible acetylation may be a regulatory posttranslational modification strategy in antibiotic-producing actinomycetes.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and reagents. All strains and plasmids used in this work are listed in Table S1 of the supplemental material. Three *Saccharopolyspora erythraea* strains, the wile-type NRRL2338 (from DSM 40517) and gene null mutant Δ*srtN* and Δ*acuA* strains, were used in this study. Two gene knockout strains were generated by protoplast transformation under polyethylene glycol (PEG) mediation and chromosome homologous recombination, replacing 771 bp of the *SacsrtN* and *SacacuA* open reading frames (ORFs) with a thiostrepton (*tsr*) resistance cassette (23). Thiostrepton-resistant clones were examined for the presence of the *SacsrtN* and *SacacuA* deletion by PCR, taking their genome as the template. The deletion was confirmed by Southern blotting and sequencing. *S. erythraea* strains were grown in the minimal medium (Evans) containing 25 mM TES [*N*-(Tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid sodium salt], 2 mM citric acid, 10 mM KCl, 0.25 mM $CaCl_2$, 1.25 mM $MgCl_2$, 2 mM Na_2SO_4, 1 mM Na_2MoO_4, 0.5% trace elements (20 μM $MnSO_4 \cdot 4H_2O$, 6 μM $ZnSO_4 \cdot 7H_2O$, 20 μM H_3BO_3, 1 μM KI, 2 μM $Na_2MoO_4 \cdot 2H_2O$, 50 μM $CuSO_4 \cdot 5H_2O$, 50 μM $CoCl_2 \cdot 6H_2O$), 2.5% (mass/vol) glucose, 2 mM NaH_2PO_4, and 10 mM $NaNO_3$ (pH 7.2). *S. erythraea* strains were also cultured in MM medium (2.5 mM L-asparagine, 1 mM K_2HPO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.035 mM $FeSO_4 \cdot 7H_2O$, pH 7.2) supplemented with 20 mM glucose or 60 mM acetate as the solo carbon resource. Aerobic 100-ml batch cultures were grown in 1-liter flasks at 37°C on a rotary shaker at 250 rpm. Cultures were inoculated to an optical density at 600 nm (OD_{600}) of 0.05 unit with exponentially growing precultures.

Protein A-conjugated agarose beads were from Amersham Biosciences. Acetyl lysine antibody (catalog number ICP0380) and acetylated bovine serum albumin (BSA; catalog number ICP6090) were from ImmuneChem Pharmaceuticals, Inc. (Burnaby, British Columbia, Canada). Trichostatin A (TSA) was purchased from Wako Chemicals.

Overproduction and purification of proteins (*Sac*AcsA, *Sac*AcuA, *Sac*SrtN, *Se*Acs, *Bs*AcsA, *Bs*AcuA, and *Se*Pat). All genes were amplified by PCR from the genomic DNA of *Saccharopolyspora erythraea*, *Salmonella enterica*, and *Bacillus subtilis*. The primers used in this work are listed in Table S2 in the supplemental material. After restriction digest, the genes coding for *Sac*AcsA (*SACE_2375*), *Sac*SrtN (*SACE_3798*), *Se*Acs (*STM4275*), and *Bs*AcsA (*Bsu29680*) were cloned into pET-28a; the genes coding for *Sac*AcuA (*SACE_5148*), *Bs*AcuA (*Bsu29690*), and *Se*Pat (*STM2651*) were cloned into pGEX-4T-2. The proteins were expressed using the *E. coli* BL21(λDE3) strain. A single colony was selected to start a 5-ml overnight culture, which was then used to inoculate 50 ml of lysogeny broth (LB) medium supplemented with 1‰ kanamycin or 1‰ ampicillin. The cells were grown at 37°C to about 0.7 OD_{600} and then induced overnight with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) at 37°C for 6 h at 20°C.

Cells were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM $KHPO_4$). The cells were disrupted by sonication, and cell debris was removed by centrifugation at 12,000 × *g* for 15 min. The resulting supernatant of *Sac*AcsA, *Sac*SrtN, *Se*Acs, and *Bs*AcsA was loaded onto a 2-ml Ni-nitrilotriacetic acid (NTA)-agarose column (Merck) that was preequilibrated with the binding buffer. After the flowthrough was discarded, the column was washed with 20 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0), and bound proteins were eluted using a linear gradient from 20 to 250 mM imidazole in 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0. The fractions were analyzed by SDS-PAGE. The *Sac*AcuA, *Bs*AcuA, and *Se*Pat proteins were purified from these cell extracts using standard glutathione affinity chromatography. Those containing the desired protein were pooled and dialyzed against buffer A (50 mM Tris and 150 mM NaCl, pH 7.5) and then concentrated using an Amicon Ultra-4 10,000-molecular-weight cutoff centrifugal device (Millipore). The protein concentration was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard.

Site-directed mutagenesis of *Sac*AcsA acetylated-site mutants. The acetylated-site mutants (K237Q, K380Q, K611Q, and K628Q) were introduced into the pET28a(+)::sacacs plasmid using a QuikChange mutagenesis kit (Stratagene) with the primers listed in Table S3 in the supplemental material. The mutations were confirmed by DNA sequencing.

***In vitro* acetyl-CoA synthetase (Acs) assays.** The specific activity of acetyl-CoA synthetase was determined at 37°C using a microplate reader (BioTek Instruments, Winooski, VT, USA) in a transparent 384-well microplate at 340 nm. The standard reaction mixture contained 100 mM Tris-HCl (pH 7.7), 10 mM L-malate (pH 7.7), 0.2 mM coenzyme A, 8 mM ATP (pH 7.5), 1 mM NAD^+, 10 mM $MgCl_2$, 3 units of malate dehydrogenase, 0.4 unit of citrate synthase, and 200 nM purified *Sac*AcsA. The reaction was started with 100 mM potassium acetate. One unit was defined as the amount of enzyme catalyzing the acetate-dependent formation of 1 mmol of NADH min^{-1} in the coupled assay (24). For determination of the K_m value, the acetate or ATP concentration in the assay was varied while the other components remained constant. The apparent steady-state kinetic parameters were estimated by nonlinear regression to fit the data to Michaelis-Menten kinetics.

***In vitro* protein acetylation assays.** To determine whether *Sac*AcsA was a substrate for *Sac*AcuA, 0.2 μM purified *Sac*AcuA protein or BSA and 5 μM purified unacetylated *Sac*AcsA protein were added to a reaction mixture (200-μl total volume) containing 0.05 M HEPES buffer (pH 7.5), 200 μM tris(2-carboxyethyl) phosphine (TCEP) hydrochloride, and 20 μM Ac-CoA. Reaction mixtures were incubated at 37°C for 2 h (15). After the reaction, the *Sac*AcsA protein samples were divided into two portions: one portion was analyzed by SDS-PAGE and Western blotting, and the other was used for measurement of the Acs activity. The acetylated *Sac*AcsA (*Sac*AcsA^{Ac}) was isolated by SDS-PAGE and then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

*Se*Acs and *Bs*AcsA (5 μM) were incubated with *Sac*AcuA (0.2 μM) and 20 μM Ac-CoA for 2 h at 37°C using the buffer system described above. The effect of *Sac*AcuA, *Se*Pat, and *Bs*AcuA on *Sac*AcsA was measured as described above using the *Sac*AcsA wild-type (*Sac*AcsA^{WT}) and mutant proteins (*Sac*Acs with a K237Q mutation [*Sac*Acs^{K237Q}], *Sac*Acs^{K380Q}, *Sac*Acs^{K611Q}, and *Sac*Acs^{K628Q}); the samples were analyzed by SDS-PAGE and Western blotting. The *Se*Acs and *Bs*AcsA acetylated by *Sac*AcuA were isolated and analyzed by LC-MS/MS as above.

***In vitro* deacetylation assays.** Samples containing 5 μM unacetylated *Sac*AcsA were first incubated with 0.2 μM *Sac*AcuA and 20 μM Ac-CoA at 37°C for 2 h. After the acetylation reaction, acetylated *Sac*AcsA was isolated from the reaction mixture by ultrafiltration and affinity chromatography. To examine whether acetylated *Sac*AcsA was deacetylated, the purified acetylated *Sac*AcsA protein was added to 50 mM HEPES (pH 8.5) buffer containing 1 mM $MgCl_2$, 1 mM NAD^+, and 0.5 μM *Sac*SrtN. The mixture was incubated at 37°C for 3 h (15). The samples were divided into two portions: one portion was resolved by SDS-PAGE and analyzed by Western blotting, and the other was used for measurement of the acetyl-CoA synthetase activity.

***In vitro* steady-state kinetic assays.** To measure the kinetic parameters of the deacetylation, the acetylated *Sac*AcsA was obtained after incubating 5 μM *Sac*AcsA with 20 μM Ac-CoA and 0.2 μM *Sac*AcuA at 37°C for 3 h. A typical deacetylation reaction mixture contained 0.1 μM *Sac*SrtN, various amounts of one substrate, and a saturating amount of the other. The reaction was initiated with the addition of *Sac*SrtN. Acs species (0.2 nmol, acetylated and nonacetylated) were withdrawn from the reaction mixture every 30 min and tested for the Acs activity as described above (15). The initial velocities of the deacetylation reaction were calculated based on the amount of active Acs generated in the indicated time. Each data point is the average of three identical assays. For the acetylation, we used a method similar to that described above. The data were fitted into the Michaelis-Menten equation to obtain the K_m and k_{cat} values.

Western blot analysis. The protein concentrations of the samples were determined using a BCA Protein Assay kit (Tiangen) with BSA as the standard. Protein samples were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane for 30 to 60 min at 100 V. The membrane was blocked at 24°C in 1× TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk (NFDM) for 2 h. Anti-acetyl-lysine (here, anti-AcK) antibody diluted 1:15,000 in TBST–0.5% NFDM was used. After incubation at 4°C for overnight, the blot was washed with TBST three times. The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1 μg/ml in TBST with 3% BSA) at ambient temperature for 2 h. An enhanced chemiluminescence (ECL) system (Pierce, USA) was used for signal detection according to the manufacturer in conjunction with a luminescent image analyzer (DNR Bio-Imaging Systems, Israel).

Purification of *Sac*AcsA from *S. erythraea*, IP, and immunoblotting (IB). Cells of *Saccharopolyspora erythraea* strains (wild type and *SacsrtN* and *SacacuA* deletion mutants) grown on different media for anti-Acs immunoprecipitation (IP) were harvested by centrifugation at 3,000 × *g* for 30 min and then ground and resuspended in PBS buffer in the presence of protease inhibitors (1 mM phenylmethanesulfonyl fluoride and Complete EDTA-free Protease Inhibitor Cocktail Tablets [Roche]) and histone deacetylase inhibitors (100 μM trichostatin A, 50 mM nicotinamide, and 50 mM sodium butyrate). The resuspended cells were disrupted by sonication, and cell debris was removed by centrifugation at 12,000 × *g* for 45 min. For immunoprecipitation, lysates (about 300 μg) were incubated with 2 μg of anti-Acs antibody (Abmart, Shanghai, China) at 4°C for 2 h, followed by the addition of 40 μl of protein A-agarose (Santa Cruz Biotechnology, Inc.) overnight. After four washes with PBS washing buffer at 4°C, bound proteins were eluted by boiling in SDS sample buffer, resolved by SDS-PAGE, and then subjected to Western blot analysis. Primary antibodies used were anti-Acs antibody and acetyl lysine antibody HRP conjugate (anti-AcK; Immunechem). Secondary antibodies were purchased from Abmart. Binding was visualized using an ECL Western blotting method. After ECL detection, films were scanned by MF-ChemiBIS software, version 3.2 (DNR Bio-Imaging Systems, Israel), and quantified with ImageJ software. The purified *Sac*AcsA from *S. erythraea* was then analyzed by LC-MS/MS spectrometry.

Mass spectrometry peptide fingerprinting. Protein digestion was performed according to the filter-aided sample preparation (FASP) procedure described by Wisniewski et al. (25). Briefly, the protein pellet (about 30 μg) was solubilized in 30 μl of SDT buffer (4% SDS, 100 mM dithiothreitol [DTT], 150 mM Tris-HCl, pH 8.0) at 90°C for 5 min. The detergent, DTT, and other low-molecular-weight components were removed using 200 μl of UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0) by multiple ultrafiltrations (Microcon units; 30 kDa). Then, 100 μl of 0.05 M iodoacetamide in UA buffer was added to block reduced cysteine residues, and the samples were incubated for 20 min in darkness. The filter was washed with 100 μl of UA buffer three times and then with 100 μl of 25 mM NH_4HCO_3 twice. Finally, the protein suspension was digested with 2 μg of trypsin (Promega) in 40 μl of 25 mM NH_4HCO_3 overnight at 37°C, and the resulting peptides were collected as a filtrate.

Tryptic digests (approximately 30 μg of predigested protein) were solid-phase extracted and analyzed by microcapillary LC (mLC)-MS/MS using a Micromass MS (Waters) and Q Exactive mass spectrometer (Thermo Finnigan, San Jose, CA) to locate protein acetylation sites. Chromatography of peptides prior to mass spectral analysis was accomplished using high-performance liquid chromatography (HPLC). Columns were made using lengths of fused silica tubing (0.15-mm outside diameter [o.d.]; 150-mm inside diameter [i.d.]) with pulled tips (1-mm orifice) that were packed with Zorbax 300SB-C_{18} peptide traps (Agilent Technologies, Wilmington, DE). An Agilent HPLC delivered solvents A (0.1% [vol/vol] formic acid in water) and B (0.1% [vol/vol] formic acid in acetonitrile [84% vol/vol], 0.1% formic acid) at either 1 ml/minute to load sample or at 150 to 200 nl/minute to elute peptides as follows: over a 50-min 4% (vol/vol) B to 50% B gradient; over another 4-min 50% (vol/vol) B to 100% B gradient; and over 6 min in 100% (vol/vol) B. As peptides were eluted from the HPLC column/electrospray source, MS/MS spectra were collected.

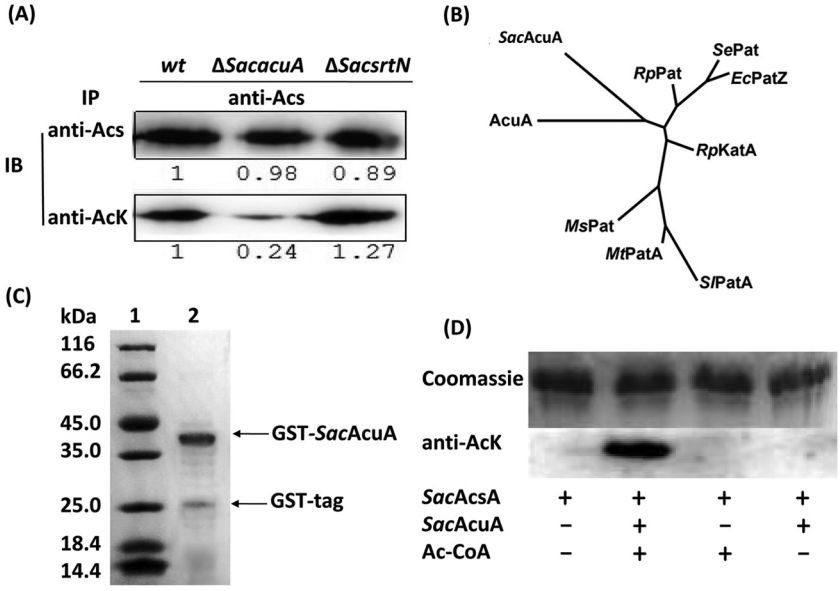

FIG 1 *Sac*AcuA enzyme is a novel protein acetyltransferase. (A) Wild-type, Δ*SacsrtN*, and Δ*SacacuA* strains of *S. erythraea* were grown in Evans minimal medium. Total protein extracts were collected, and the acetyl-CoA synthetase *Sac*AcsA was immunoprecipitated with anti-Acs antibody and subjected to Western blot analysis. Immunoblotting was performed with anti-*Sac*Acs and anti-AcK antibodies. The band intensities were quantified by densitometry using ImageJ software. (B) The phylogenetic analysis was conducted (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi) with *Sac*AcuA and the following eight protein acetyltransferases: *Se*Pat (STM2651), *Rp*Pat (RPA4240), *E. coli* PatZ (*Ec*PatZ) (b2584), *Sl*PatA, *Ms*Pat, *Mt*PatA, *Bs*AcuA, and *Rp*KatA (rpa3031). (C) SDS-PAGE analysis of purified recombinant *Sac*AcuA. Lane 1, molecular mass marker; lane 2, purified glutathione *S*-transferase (GST)-*Sac*AcuA. (D) The purified *Sac*AcsA was *in vitro* incubated with or without *Sac*AcuA and Ac-CoA at 37°C for 2 h. After incubation, samples were collected and analyzed by SDS-PAGE, and the acetylation levels were determined by Western blotting using specific anti-AcK antibody.

MS/MS spectra were searched using the MASCOT engine (version 2.2; Matrix Science, London, United Kingdom) against the Uniprot Saccharopolyspora_NRRL23338 database (7,165 sequences; accessed 1 July 2013). For protein identification, the following options were used: peptide mass tolerance, 20 ppm; MS/MS tolerance, 0.1 Da; enzyme, trypsin; missed cleavage, 2; fixed modification, carbamidomethyl (C); variable modification, oxidation (M) and acetylation (K, N-terminal); decoy database pattern, reverse. All reported data were based on 99% confidence for protein identification as determined by a false discovery rate (FDR) of ≤1%.

RESULTS AND DISCUSSION

The *Sac*AcuA enzyme of *Saccharopolyspora erythraea* is a novel protein acetyltransferase. It was reported that the activity of acetyl-CoA synthetase was modulated by acetylation in some bacteria (5, 6, 12, 13). To investigate whether acetyl-CoA synthetase from the erythromycin-producing *S. erythraea* (*Sac*AcsA) is acetylated *in vivo*, immunoprecipitation (IP) and immunoblotting (IB) analyses were conducted to directly test the acetylation status of *Sac*AcsA. *Sac*AcsA from *S. erythraea* cells was immunoprecipitated with an antibody to *Sac*AcsA (encoded by *SACE_2375*). Acetyl-lysine levels were detected on *Sac*AcsA immunoprecipitates with anti-AcK antibody. As expected, the results demonstrated that *Sac*AcsA was acetylated *in vivo* (Fig. 1A). The *S. erythraea* genome contains 42 genes putatively encoding GNAT protein acetyltransferases. Significantly, no *Se*Pat homologue was found. We found that antibody against AcK reacted strongly with the *Sac*AcsA enzyme isolated from a wild-type strain; in contrast, the antibody exhibited weak reactivity against *Sac*AcsA isolated from a strain devoid of *Sac*AcuA (Δ*SacacuA* strain) (Fig. 1A). Because the acetylation level of *Sac*AcsA was significantly reduced in the Δ*SacacuA* strain, we surmised that the *Sac*AcuA might be involved in the acetylation of *Sac*AcsA. The *SacacuA* gene encodes a single-domain GNAT protein acetyltransferase (155 amino acids; referred to as *Sac*AcuA), which is 31% identical to the *B. subtilis* AcuA enzyme (*Bs*AcuA). Furthermore, a phylogenetic analysis with the full-length sequence of *Sac*AcuA and the eight protein acetyltransferases for lysine acetylation showed that *Sac*AcuA clustered with *Bs*AcuA (Fig. 1B) (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi), thus suggesting that *Sac*AcuA could acetylate acetyl-CoA synthetases in *S. erythraea* as *Bs*AcuA does in *B. subtilis*.

Additionally, the comparison of amino acid sequences of *Sac*AcuA and *Bs*AcuA revealed that *Sac*AcuA contains four conserved motifs, sequentially labeled C, D, A, and B, found in GNAT family members (see Fig. S1in the supplemental material). Motif A, as the core of the GNAT domain, is the most highly conserved motif and generally has an R/Q-X-X-G-X-G/A sequence that is important for acetyl-CoA recognition and binding. The $\mathbf{R}_{GS}\mathbf{G}_{V}\mathbf{A}$ sequence (conversed residues in boldface) is indeed found in motif A of *Sac*AcuA while it is not observed in *Bs*AcuA. The SWISS-MODEL server of the Protein Model Portal (PMP [http://www.proteinmodelportal.org/?pid=modeling_interactive]) was used online for model building for the structure of *Sac*AcuA (see Fig. S2 in the supplemental material). Further, we confirmed that *Sac*AcuA directly acetylated *Sac*AcsA *in vitro* by incubating purified *Sac*AcuA (Fig. 1C) with Ac-CoA and recombinant *Sac*AcsA of *S. erythraea*. As shown in Fig. 1D, the results showed that *Sac*AcsA was a substrate of *Sac*AcuA.

To test the effect of acetylation on enzyme activity, *Sac*AcsA was incubated with *Sac*AcuA in the presence or absence of Ac-CoA for 2 h. In the presence of both Ac-CoA and *Sac*AcuA, *Sac*AcsA activity was reduced >80%, indicating that *Sac*AcuA

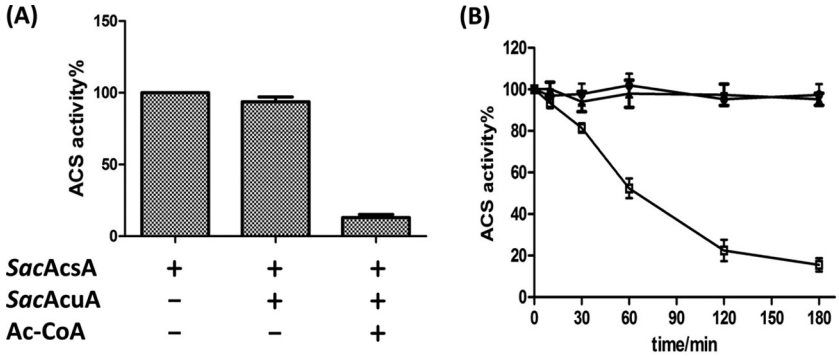

FIG 2 Effects of acetylation on *Sac*AcsA activity. (A) *In vitro* acetylation affected the activity of *Sac*AcsA. The enzyme activity of *Sac*AcsA after incubation with or without Ac-CoA in the presence of *Sac*AcuA was measured every 30 min. The *Sac*AcsA activity is described as a percentage of the maximum activity determined for *Sac*AcsA before acetylation. Data are expressed as means plus standard deviations of three identical assays. (B) Time-dependent inactivation of *Sac*AcsA by acetylation. *Sac*AcsA activity was measured at different time intervals during incubation with *Sac*AcuA. □, 0.2 μM *Sac*AcuA and 20 μM Ac-CoA; ○, 0.2 μM *Sac*AcuA; △, 5 μM *Sac*AcsA. Each data point represents the average from three independent assays.

lysine acetylation effectively decreased *Sac*AcsA activity (Fig. 2A). Time-dependent inactivation of *Sac*AcsA by *Sac*AcuA acetylation was also investigated. Data presented in Fig. 2B show that *Sac*AcsA gradually lost its activity during acetylation by the *Sac*AcuA enzyme.

To measure the kinetic parameters of the *Sac*AcuA-catalyzed acetylation reaction of *Sac*AcsA, we used a coupled enzymatic assay to monitor the acetylation reaction continuously. The resulting data were fitted using the Michaelis-Menten kinetics model. The kinetic parameters of *Sac*AcuA were compared with those of *Bs*AcuA from *Bacillus subtilis* and Gcn5 from yeast. The results are shown in Table 1. The K_m and k_{cat} values for Ac-CoA are 56 μM and 0.05 s^{-1}, respectively. This is comparable to the intracellular levels reported for acetyl-CoA, which can reach cytoplasmic concentrations of 20 to 600 μM in *E. coli* (26). Similarly, The K_m and k_{cat} values for *Sac*AcsA are 3.9 μM and 0.02 s^{-1}. *Sac*AcuA exhibited a K_m value of 56 μM for Ac-CoA substrate, which was slightly higher than that of *B. subtilis Bs*AcuA (K_m of 22 μM) (27), which was 22-fold higher than that of the yeast Gcn5 histone acetyltransferase (HAT) (K_m of 2.5 μM) (28). These results indicated that *Sac*AcuA was active at high intracellular levels of Ac-CoA. The k_{cat} of *Sac*AcuA for Ac-CoA was 0.05 s^{-1}, a turnover number that was 34-fold lower than the k_{cat} value (1.7 s^{-1}) of the yeast Gcn5 HAT and 6-fold lower than the k_{cat} value (0.3 s^{-1}) of *B. subtilis Bs*AcuA.

TABLE 1 Kinetic analysis of *Sac*AcuA and *Sac*SrtN

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
*Sac*AcuA	Ac-CoA	56 ± 1	0.049 ± 0.003	860 ± 51
	AcsA	3.9 ± 0.7	0.019 ± 0.001	(5.2 ± 0.9) × 10^3
*Bs*AcuA[a]	Ac-CoA	22 ± 2	0.3	1.4 × 10^4
	Peptide	20 ± 2	0.3	1.5 × 10^4
Yeast Gcn5[a]	Ac-CoA	2.5 ± 1.4	1.7 ± 0.12	6.8 × 10^5
	H3 peptide	490 ± 80	1.7 ± 0.12	3.5 × 10^3
*Sac*SrtN	Ac-AcsA	52 ± 5	0.033 ± 0.001	590 ± 72
	NAD^+	240 ± 15	0.052 ± 0.002	220 ± 31

[a] Data are from previously published literature. The peptide substrate of *Bs*AcuA was the C-terminal 31 residues of the *Bs*AcsA protein (LPKTRSG**K**IMRRVLKAWELNLPAG DLSTMED) (27). The H3 peptide substrate of Gcn5 was ARTKQTARKSTGG**K**APPKQ LC, corresponding to the 20 amino-terminal residues of human histone H3 and an additional carboxyl-terminal cysteine (28). Boldface indicates acetylated lysine.

***In vitro*, *Sac*AcsA is acetylated at four lysine residues.** To determine the acetylation sites of *Sac*AcsA protein, we cloned the *SacacsA* gene and purified recombinant *Sac*AcsA (668 amino acids). *Sac*AcsA was incubated with *Sac*AcuA and Ac-CoA for 2 h. The *in vitro*-acetylated *Sac*AcsA protein was subjected to trypsin digestion, and the resulting peptides were analyzed by tandem mass spectrometry. Lysine-acetylated peptides may be identified as they have a mass increment of 42 Da compared with unacetylated peptides. Four peptides were acetylated, the sequences of which are T**K**(237)TDVEWNDGR, TFM**K**(380)WGAEIPAR, DH VAHEIGPIA**K**(611)PR, and SG**K**(628)IMR (see Fig. S3 in the supplemental material). Lysine 237, lysine 380, lysine 611, and lysine 628 (in boldface) were identified as the acetylated residues in *in vitro*-acetylated *Sac*AcsA (Fig. 3A). In our study, multiple acetylation sites were observed in *S. erythraea Sac*AcsA, but three of these sites (K237, K380, and K611) were not identified in any of the other studies, and these lysines were not conserved in Acs homologues in other organisms (Fig. 3B). In *S. erythraea Sac*AcsA, K628 is a conserved active-site residue (conserved putative acylation motif PXXXXG**K**), similar to Lys609 of *Se*Acs from *S. enterica*, Lys606 of *Rp*Acs from *R. palustris*, Lys617 of *Mt*Acs from *M. tuberculosis*, and Lys549 of *Bs*AcsA from *B. subtilis*. K237 and K380 lysine residues are located in the CoA-binding domain and acetate-binding domain.

To confirm the sites of acetylation and investigate the effects of these residues on the acetylation level of *Sac*AcsA, we created substitution mutations at these positions to generate K237Q, K380Q, K611Q, and K628Q variants of *Sac*AcsA (see Fig. S4 in the supplemental material). Glutamine (Q) abolishes the positive charge and serves as a structural mimic for acetyl-lysine. *Sac*AcsA^{K237Q}, *Sac*AcsA^{K380Q}, *Sac*AcsA^{K611Q}, *Sac*AcsA^{K628Q}, and *Sac*AcsA^{WT} were incubated with *Sac*AcuA enzyme and Ac-CoA. Western blotting was performed to detect the acetylation level of these AcsA variants with anti-AcK antibody. As shown in Fig. 3C, all *Sac*AcsA variants were acetylated by *Sac*AcuA, indicating that multiple *Sac*AcsA lysine residues were modified by *Sac*AcuA, in agreement with the results obtained mass spectrometry. *Sac*AcsA^{K237Q}, *Sac*AcsA^{K380Q}, *Sac*AcsA^{K611Q}, and *Sac*AcsA^{K628Q} mutants revealed

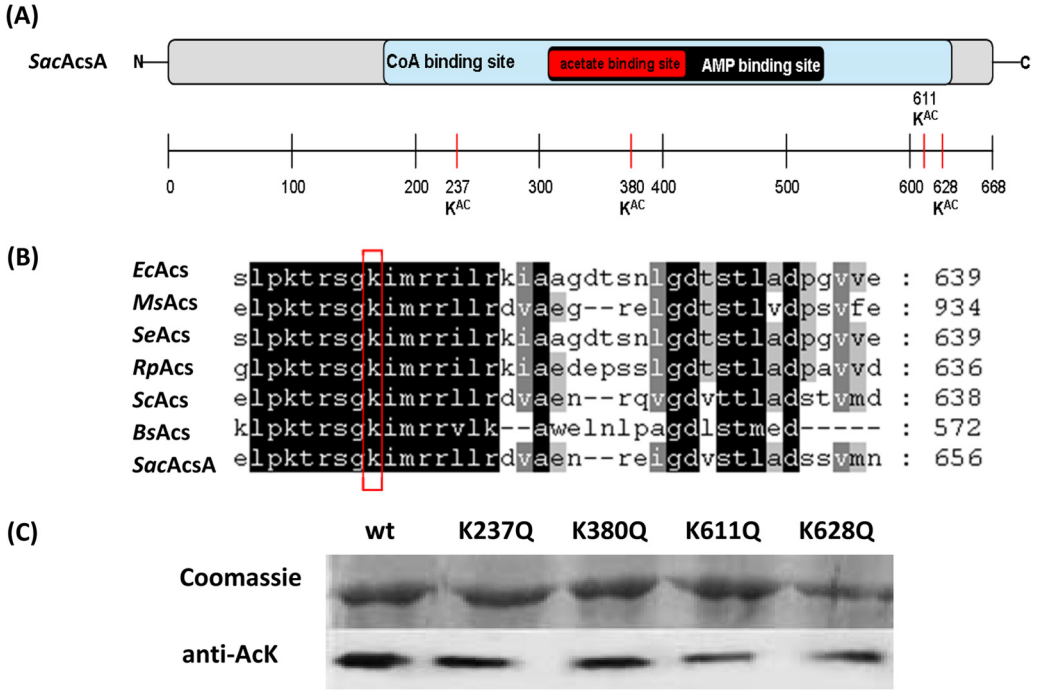

FIG 3 Acetylation sites of *Sac*AcsA. (A) Predicted domains of *Sac*AcsA are shown in differently colored boxes. Red lines show the positions of acetylated lysine residues. (B) Multiple sequence alignment of the conserved putative acylation motif in acetyl-CoA synthetases from different species. The active-site lysine of all species is boxed in red. (C) The effect of *Sac*AcuA on *Sac*AcsA wild type and four variants (*Sac*Acs^{K237Q}, *Sac*Acs^{K380Q}, *Sac*Acs^{K611Q}, and *Sac*Acs^{K628Q}). Unacetylated *Sac*AcsA wild type and four variants were incubated with *Sac*AcuA and Ac-CoA at 37°C for 2 h. After incubation, samples were collected and analyzed by SDS-PAGE, and the acetylation levels were determined by Western blotting.

similar acetylation levels, which were decreased slightly compared with the level of *Sac*AcsA^{WT}.

For comparison, *Sac*AcsA^{WT} and four variants were also incubated with *Se*Pat from *S. enterica* and *Bs*AcuA from *B. subtilis*. *Se*Pat and *Bs*AcuA acetylated *Sac*AcsA^{WT}, *Sac*AcsA^{K237Q}, *Sac*AcsA^{K380Q}, and *Sac*AcsA^{K611Q} but did not acetylate *Sac*AcsA^{K628Q}, suggesting that *Se*Pat and *Bs*AcuA modified only the conserved lysine residue K628 in the active site (Fig. 4A). No acetylation was identified at the three residues K237, K380, and K611 in *Sac*AcsA. As shown in Fig. 4B, *Sac*AcuA enzyme can also acetylate *Se*Acs from *S. enterica* and *Bs*AcsA from *B. subtilis*. However, *Se*Acs and *Bs*AcsA revealed similar acetylation levels, but these acetylation levels were decreased drastically compared with the *Sac*AcsA level, indicating that *Se*Acs and *Bs*AcsA were poor substrates for *Sac*AcuA. To further investigate whether *Sac*AcuA modified the multiple lysine residues in other acetyl-CoA synthetases, we determined the acetylation sites of *Se*Acs and *Bs*AcsA modified by *Sac*AcuA. The *in vitro*-acetylated acetyl-CoA synthetases were digested with trypsin, and the resulting peptides were analyzed by tandem mass spectrometry. The results showed that lysine residues 29, 44, 56, 585, and 609 (in boldface) were acetylated in peptides Y**K**(29)QSINDPDTFWGEQGK, QSINDPDTFWGEQG**K**(44)ILDWITPYQK, V**K**(56)NTSFAPGNVSIK, **K**(585)EIGPLATPDVLHWTDSLPK, and SG**K**(609)IMR of *Se*Acs (see Fig. S5 in the supplemental material); lysine residues 16, 98, 320, 524, and 549 were acetylated in peptides ALPAIEGDHNL**K**(16)N

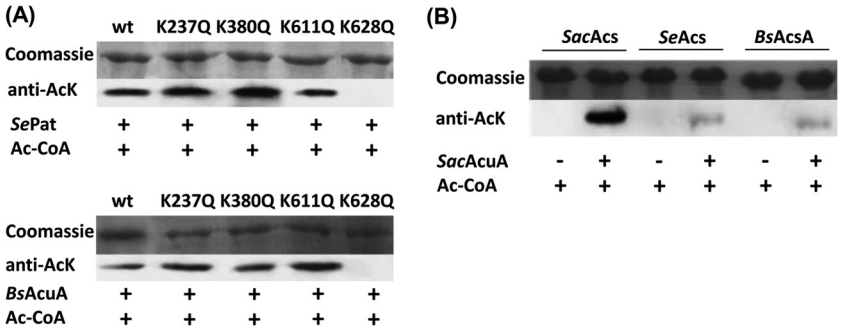

FIG 4 *Se*Pat and *Bs*AcuA acetylate only one lysine residue. (A) The effect of *Se*Pat and *Bs*AcuA on *Sac*AcsA wild type and four variants (*Sac*Acs^{K237Q}, *Sac*Acs^{K380Q}, *Sac*Acs^{K611Q}, and *Sac*Acs^{K628Q}). Unacetylated *Sac*Acs wild type and four variants were incubated with *Se*Pat or *Bs*AcuA in the presence of Ac-CoA at 37°C for 2 h. After incubation, samples were collected and analyzed by SDS-PAGE, and the acetylation levels were determined by Western blotting. (B) *Sac*AcuA acetylated *Sac*AcsA, *Se*Acs, and *Bs*AcsA. After incubation with *Sac*AcuA and Ac-CoA at 37°C for 2 h, samples were analyzed by SDS-PAGE and Western blotting.

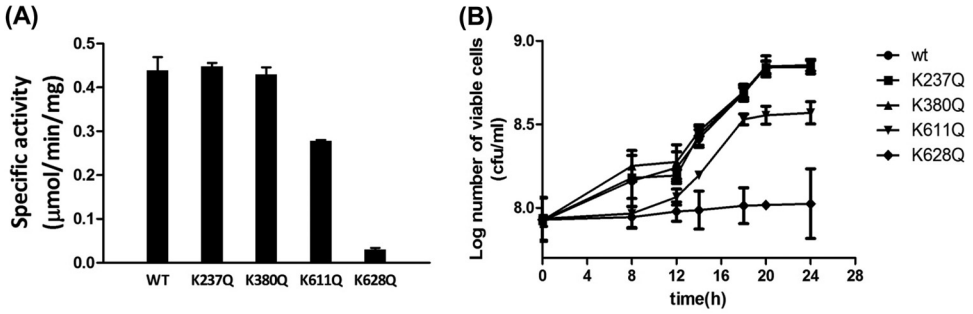

FIG 5 Effect of acetylated sites on enzyme activity. (A) The activity of AcsA (50 pM) incubated with ATP (8 mM), CoA (0.2 mM), and 100 mM acetate at 37°C. (B) Growth curves of the *S. enterica* Δ*acs* strains complemented with *Sac*AcsA wild-type or mutant genes in acetate minimal medium (10 mM acetate as a sole carbon source). Expression of the *acs* gene was induced by addition of L-(+)-arabinose (250 μM). Cell density measurements at 600 nm were acquired. Graphed points represent the means of three independent measurements.

YEETYR, YGNVE**K**(98)GDR, MLMGAGDEMAA**K**(320)YDLT SLR, LFV**K**(524)QGLAAHAAPR, and SG**K**(549)IMR of *Bs*AcsA (see Fig. S6 in the supplemental material). It was reported that only the conserved active-site residue of acetyl-CoA synthetase (Lys609 of *Se*Acs or Lys549 of *Bs*AcsA) was acetylated by cognate acetyltransferases in *S. enterica* and *B. subtilis* (5, 12). The acetyltransferase *Sac*AcuA appears to have evolved to acetylate multiple lysine residues, unlike other previously reported bacterial acetyltransferases which acetylate only one lysine residue in the conserved putative acylation motif PXXXXG**K** in AMP-forming acyl-CoA synthetases.

The previously reported bacterial acetyltransferases exhibit the specificity of lysine sites for protein acetylation. There is the proposed acetylation motif (PXXXXGK) found in AMP-forming acyl-CoA synthetases (20). GNAT acetyltransferases recognize this motif and acetylate the last lysine residue of PXXXXG**K**. It is unclear why these acetyltransferases acetylate a lysine only in a specific position. We speculate that such site specificity is attributed to other functional domains of multidomain acetyltransferases or a substrate-binding cleft composed of motifs A and D. As shown in Fig. S1 in the supplemental material, motif C is involved in orienting the protein substrate, and motif D forms part of the β-sheet core and, together with motif A, makes up half of the catalytic site (29). There are more α-helixes and β-sheets in the core region of *Bs*AcuA, such as α3, β6, α4, β7, α7, and α8, and this region forms a substrate binding pocket for the PXXXXGK motif and therefore likely plays a crucial role in determining substrate specificity. In *Sac*AcuA, the absence of such α-helixes and β-sheets maybe leaves larger or more flexible cleft benefits for the access of the variant lysine sites of substrates to be acetylated. Thus, *Sac*AcuA has broad specificity for lysine acetylation, allowing it to acetylate multiple lysine residues. The substrate recognition and acetylation mechanism of specialized lysine sites by acetyltransferases remain to be determined.

***Sac*AcsA is inactive mainly upon acetylation of K628.** To examine the effects of acetylation lysine residues on *Sac*AcsA activity, the activities of *Sac*AcsA^{WT} and *Sac*AcsA variants were determined. As shown in Fig. 5A, two *Sac*AcsA variants were less active to some extent, while lysine acetylation significantly inhibited *Sac*AcsA activity. The results showed that mutations at the K238 or K380 site produced activity similar to that of the *Sac*AcsA^{WT}, while mutation of K611 resulted in a 30% decrease in acetyl-CoA synthetase activity compared to the wild-type enzyme. In contrast, the K628Q mutant was essentially inactive, with <10% of the wild-type *Sac*AcsA activity. These results indicate that residue K628 is critical for catalytic activity. Substitution of K628 effectively abolished activity of AcsA, in agreement with the results that acetylation of K628 inhibited *Sac*AcsA activity.

To test the effects of substitutions at the four different Lys residues in *Sac*AcsA *in vivo*, alleles encoding wild-type and variant *Sac*AcsA proteins were introduced into an *S. enterica* Δ*acs* strain carrying a chromosomal deletion of the *acs* gene. The resulting strains were grown at 37°C in 5 ml of the minimal acetate medium (10 mM acetate as a sole carbon source), and growth was monitored in triplicate at an OD_{600} (30). As shown in Fig. 5B, differences in growth behavior were observed. For example, the strain synthesizing *Sac*AcsA^{K628Q} failed to grow on 10 mM acetate as a sole carbon and energy source; growth of the strain that synthesized *Sac*AcsA^{K611Q} was strongly reduced, reaching only 60% of the cell density of the strain synthesizing *Sac*AcsA^{WT}; strains that synthesized *Sac*AcsA^{K237Q} or *Sac*AcsA^{K380Q} grew as well as the strain synthesizing *Sac*AcsA^{WT}. The *in vivo* experimental results were consistent with the activity of the *Sac*AcsA variants measured *in vitro* (Fig. 5A).

To further investigate the mechanism by which each acetylation site reduces *Sac*AcsA activity, enzyme kinetic studies of the *Sac*AcsA^{WT} and its variants were performed. The results are shown in Table 2. The mutants at the K237, K380, and K611 sites exhibited k_{cat} values of 35 to 40 s^{-1}, which were very similar to the turnover number for the wild-type enzyme (33.5 s^{-1}). The k_{cat} values of *Sac*AcsA^{K628Q} was 2.7 s^{-1}, about 12.4-fold lower than the value for *Sac*AcsA^{WT}. These results indicated that it was the K628 site, not K237, K380, or K611, that was directly involved in enzyme catalytic activity. The crystal structure of benzoyl-CoA synthetase from *Burkholderia xenovorans* (RCSB Protein Data Bank

TABLE 2 Kinetic analysis of WT *Sac*AcsA and its variants

*Sac*AcsA enzyme	K_m (acetate [mM])	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
WT	3.3 ± 0.3	33.5 ± 6.2	(1.0 ± 0.3) × 10^4
K237Q	3.3 ± 0.2	37.5 ± 1.2	(1.0 ± 0.1) × 10^4
K380Q	2.8 ± 0.5	40.1 ± 1.4	(1.5 ± 0.3) × 10^4
K611Q	7.3 ± 0.1	34.8 ± 3.4	(5.0 ± 0.5) × 10^3
K628Q	18.1 ± 0.1	2.7 ± 0.1	(1.5 ± 0.1) × 10^2

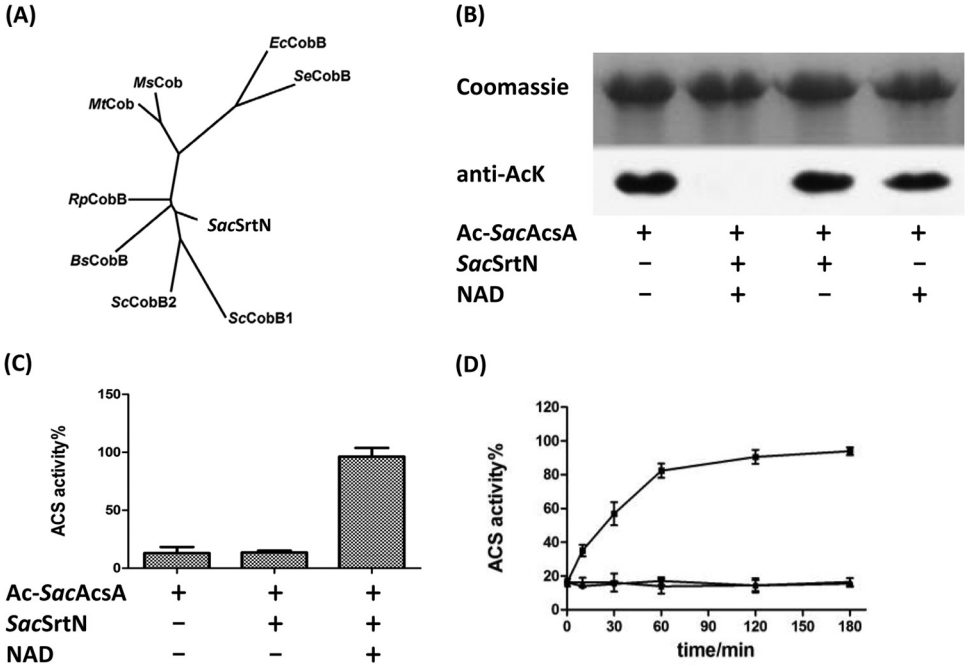

FIG 6 *Sac*SrtN deacetylated acetylated acetyl-CoA synthetase. (A) Phylogenetic analysis with *Sac*SrtN and the eight protein deacetylases: *Se*CobB (STM1221), *Rp*SrtN (formerly rpa2524), *Ec*CobB (b1120), *Ms*CobB (MSMEG_5175), *Mt*CobB (Rv1151c), *Sc*CobB1 (SCO0452), *Sc*CobB2 (SCO6464), and *Bs*SrtN (*yhdZ*; BSU09650). (B) Deacetylation of *Sac*AcsA by *Sac*SrtN. Acetylated *Sac*AcsA was incubated with or without *Sac*SrtN and 1 mM NAD^+ at 37°C for 3 h. After incubation, samples were collected and analyzed by SDS-PAGE, and the acetylation levels were determined by Western blotting using specific anti-AcK antibody. (C) Effect of deacetylation on the activity of *Sac*AcsA. Data are expressed as means plus standard deviations of three repeated assays. (D) Time-dependent reactivation of acetylated *Sac*AcsA by deacetylation. *Sac*AcsA activity was measured at different time intervals during the incubation with *Sac*SrtN. ■, 0.5 μM *Sac*SrtN and 1 mM NAD^+; ●, 0.5 μM *Sac*SrtN; ▲, 5 μM acetylated *Sac*AcsA. Each data point represents the average from three repeated assays.

[PDB] 2V7B) shows that a conserved lysine residue (such as K628) in its active site forms two hydrogen bonds with the carboxylate group of the substrate and orients the acid substrate in the active site. This process is critical to the first step of the *Sac*AcsA catalysis reaction, which ordinarily consumes ATP to form an acyl-adenylate intermediate, releasing pyrophosphate. The presence of the acetyl moiety would block the interactions between the ε-amino group of lysine and the carboxylate group of the substrate (31). Thus, Lys628 in a putative acylation motif was shown to be important for catalysis of the overall reaction. The K628Q and K611Q mutants increased K_m values for acetate (5.5-fold and 2.2-fold increases, respectively), compared with the wild-type protein, whereas the mutants at the K237 and K380 sites revealed K_m values similar to the value of the wild-type protein. The above observations indicate a possible role of K628 and K611 in binding the acetate substrate. It is worth noting that the K611 mutants had no obvious effect on the k_{cat} value but increased the K_m value, suggesting that K611 may be involved in binding acetate but is not needed for catalysis. Kinetic analysis of the K628Q variants revealed a significantly reduced catalytic efficiency (k_{cat}/K_m) value for acetate (67-fold decrease).

***Sac*AcsA^{Ac} is deacetylated and reactivated by the sirtuin-type deacetylase *Sac*SrtN.** Acetylation of acetyl-CoA synthetase is reversed by deacetylases. It has been found that there are two types of protein deacetylases responsible for deacetylation of proteins in prokaryotes. Bacterial sirtuin-like deacetylases, reported first in *S. enterica* (5), use NAD^+ as the substrate to deacetylate proteins. In so doing, sirtuins transfer the acetyl group from the protein to ADP-ribose, producing nicotinamide and 2′-*O*-acetyl-ADP-ribose as products. The other type of AcuC-like deacetylase, reported first in *B. subtilis* (12), is a zinc-dependent member of the class IIa histone deacetylases that uses water to hydrolyze the acetyl moiety, releasing acetate, as seen in reactions catalyzed by *Bs*AcuC and *Rp*LdaA. Bioinformatics analysis of the *S. erythraea* genome identified the gene *SACE_3798* (predicted to encode a 259-residue protein, here referred to as *Sac*SrtN) as homologous to the *S. enterica cobB* (35%), which encodes an NAD^+-dependent deacetylase sirtuin. We also found gene *SACE_1779*, which encodes a putative zinc-dependent deacetylase enzyme (predicted to be 391 amino acids long) that is homologous to the *B. subtilis acuC* gene (40%). A phylogenetic analysis with the full-length sequence of *Sac*SrtN and the eight CobB-like deacetylases previously reported showed that *Sac*SrtN clusters with the *Streptomyces coelicolor* CobB1 (*Sc*CobB1) and *Sc*CobB2 (Fig. 6A).

Previous works demonstrated that the *S. enterica Se*CobB sirtuin deacetylated efficiently and reactivated acetylated acetyl-CoA synthetase (5). To assess the deacetylase activity of *Sac*SrtN, *S. erythraea* AcsA (*Sac*AcsA) was acetylated by *Sac*AcuA with acetyl-CoA, and the product of the reaction was used as the substrate for *in vitro* deacetylation activity assays. The acetylated *Sac*AcsA was incubated with *Sac*SrtN in the absence and presence of NAD^+. The acetylation level of *Sac*AcsA was monitored by Western blotting using antibody against acetyl-lysine (Fig. 6B). In the presence of *Sac*SrtN and NAD^+, the acetylation level of *Sac*AcsA was found to be significantly decreased (almost undetectable), indicating that *Sac*SrtN removed acetyl groups from acetyl-lysine residues of *Sac*AcsA in an NAD^+-dependent manner. These results also showed that *Sac*AcsA acetylation was reversible. After deacetyla-

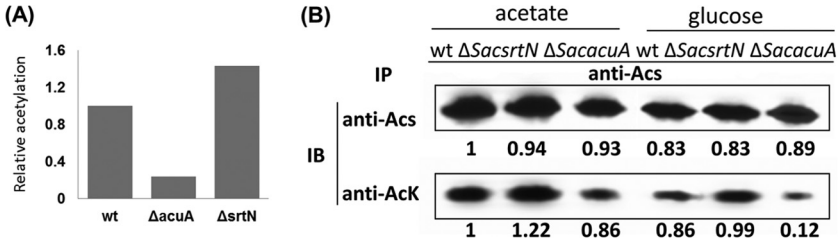

FIG 7 Analysis of *Sac*AcsA acetylation *in vivo*. (A) Relative acetylation of the results shown in Fig. 1A. (B) WT, Δ*SacsrtN*, and Δ*SacacuA* strains of *Saccharopolyspora erythraea* expressing *Sac*AcsA were grown in minimal medium with acetate or glucose as a carbon source. Culture samples were collected as a function of time, cells were analyzed, and *Sac*AcsA was immunoprecipitated with anti-*Sac*AcsA antibody and subjected to Western analysis. Immunoblotting was performed with anti-*Sac*AcsA and anti-AcK antibodies. Representative blots are shown. The band intensities were quantified by densitometry using ImageJ software.

tion, *Sac*AcsA activity was restored to a level comparable to that of the nonacetylated *Sac*AcsA control (Fig. 6C). Time-dependent activation of *Sac*AcsA by *Sac*SrtN deacetylation was also studied. As shown in Fig. 6D, an increase in *Sac*AcsA activity over time was observed during deacetylation by the *Sac*SrtN enzyme. The kinetic parameters of *Sac*SrtN for deacetylation of acetylated *Sac*AcsA were determined. The resulting data are shown in Table 1. The *Km* and k_{cat} values for *Sac*AcsA^{Ac} were 52 μM and 0.03 s^{-1}. Similarly, The K_m and k_{cat} values for NAD^+ were 240 μM and 0.05 s^{-1}.

***Sac*AcsA is also acetylated *in vivo* at multiple lysine residues.** We have provided biochemical evidence to show that *Sac*AcsA is a substrate of *Sac*AcuA and *Sac*SrtN *in vitro*; however, it was important to determine whether *Sac*AcsA could be utilized as a substrate of *Sac*AcuA and *Sac*SrtN *in vivo*. To investigate whether *Sac*AcsA was posttranslationally modified *in vivo*, we performed an immunoprecipitation experiment to assess the acetylation state of *Sac*AcsA in *S. erythraea*. *Sac*AcsA isolated from *S. erythraea* was subjected to trypsin digestion, and the resulting peptides were analyzed by LC-MS/MS. Sequence determination of the peptides identified K237, K380, K611, and K628 (in boldface) as multiple sites of acetylation of *Sac*AcsA. MS/MS spectra of the peptides T**K**(237)TDVEWNDGR, TFM**K**(380)WGAEIPAR, DHVAHEIG PIA**K**(611)PR, and SG**K**(628)IMR are shown in Fig. S7 in the supplemental material, in agreement with the results obtained from *in vitro* acetylation experiments. Based on these data, we inferred that, *in vivo*, *Sac*AcsA was acetylated at multiple sites by the *Sac*AcuA acetyltransferase.

***In vivo*, *Sac*AcsA acetylation is influenced by extracellular nutrient availability or lack of enzymes.** To provide more direct evidence for acetylation and deacetylation of endogenous *Sac*AcsA by *Sac*AcuA and *Sac*SrtN *in vivo*, we also analyzed the acetylation status of endogenous *Sac*AcsA in three strains (the wild-type, Δ*SacacuA*, and Δ*SacsrtN* strains). Immunoprecipitation experiments were conducted with 300-μg protein samples from three strains. Anti-*Sac*AcsA antibody was used to monitor the amount of *Sac*AcsA enzyme. Immunoblotting with anti-acetyl-lysine antibody showed that the acetylation level of endogenous *Sac*AcsA in the Δ*SacacuA* (Δ*SACE_5148*) strain was much lower than that in the wild-type and Δ*SacsrtN* (Δ*SACE_3798*) strains (Fig. 1A and 7A). *Sac*AcsA amounts were comparable in all three strains (Fig. 1A), indicating that endogenous *Sac*AcsA is acetylated *in vivo* and that it may be a substrate for the *Sac*AcuA acetyltransferase and *Sac*SrtN deacetylase.

*Sac*AcsA is involved in carbon metabolism. We therefore investigated the acetylation of *Sac*AcsA under culture conditions with acetate or glucose as carbon and energy sources. Endogenous *Sac*AcsA enzymes were immunoprecipitated from *S. erythraea* wild-type, Δ*SacacuA*, and Δ*SacsrtN* strains grown in minimal medium supplemented with acetate or glucose. The acetylation state of *Sac*AcsA was assessed by immunoblotting with anti-AcK antibody (Fig. 7B). We found that the amount of Acs enzyme in three strains in the presence of acetate increased slightly compared with culture in the presence of glucose. The level of *Sac*AcsA acetylation also revealed a similar pattern. It is worth noting that inactivation of *Sac*AcuA (in the Δ*SacacuA* strain) led to a significant decrease of acetylation in glucose-grown cells.

In summary, we identified the Gcn5-like protein acetyltransferase *Sac*AcuA responsible for acetylation of acetate-scavenging acetyl-CoA synthetase *Sac*AcsA in *S. erythraea*. Acetylated AcsA then is deacetylated by a sirtuin-type NAD-dependent deacetylase *Sac*SrtN. *In vitro* acetylation/deacetylation of *Sac*AcsA enzyme was verified by Western blotting, mass spectrometry, and gene knockout (Δ*SacacuA*), and acetylated lysine residues including Lys^{237}, Lys^{380}, Lys^{611}, and Lys^{628} were identified. The acetyltransferase *Sac*AcuA revealed a unique feature that can acetylate multiple lysine sites, unlike other previously reported bacterial acetyltransferases which acetylate only one lysine residue in the conserved putative acylation motif PXXXXGK in AMP-forming acyl-CoA synthetases. The activity of *Sac*AcsA is controlled by lysine acetylation. Site-specific mutagenesis experiments were conducted to investigate the effects of the acetylated lysine residues on the activity of *Sac*AcsA. The immunoprecipitation data showed that *in vivo* acetylation of *Sac*AcsA is influenced by glucose and acetate availability. These results suggest that reversible acetylation may also be a conserved regulatory PTM strategy in antibiotic-producing actinomycetes.

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