

## In *Bacillus subtilis*, the Sirtuin Protein Deacetylase, Encoded by the *srtN* Gene (Formerly *yhdZ*), and Functions Encoded by the *acuABC* Genes Control the Activity of Acetyl Coenzyme A Synthetase<sup>∇†</sup>

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**This report provides in vivo evidence for the posttranslational control of the acetyl coenzyme A (Ac-CoA) synthetase (AcsA) enzyme of *Bacillus subtilis* by the *acuA* and *acuC* gene products. In addition, both in vivo and in vitro data presented support the conclusion that the *yhdZ* gene of *B. subtilis* encodes a NAD<sup>+</sup>-dependent protein deacetylase homologous to the yeast Sir2 protein (also known as sirtuin). On the basis of this new information, a change in gene nomenclature, from *yhdZ* to *srtN* (for sirtuin), is proposed to reflect the activity associated with the YdhZ protein. In vivo control of *B. subtilis* AcsA function required the combined activities of AcuC and SrtN. Inactivation of *acuC* or *srtN* resulted in slower growth and cell yield under low-acetate conditions than those of the wild-type strain, and the *acuC srtN* strain grew under low-acetate conditions as poorly as the *acsA* strain. Our interpretation of the latter result was that both deacetylases (AcuC and SrtN) are needed to maintain AcsA as active (i.e., deacetylated) so the cell can grow with low concentrations of acetate. Growth of an *acuA acuC srtN* strain on acetate was improved over that of the *acuA<sup>+</sup> acuC srtN* strain, indicating that the AcuA acetyltransferase enzyme modifies (i.e., inactivates) AcsA in vivo, a result consistent with previously reported in vitro evidence that AcsA is a substrate of AcuA.**

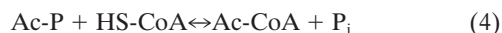
Acetate is abundant in soil, the natural habitat of the gram-positive bacterium *Bacillus subtilis*. This short-chain fatty acid accumulates in soil and other environments because it is the product of many fermentative processes (7). Many prokaryotes use acetate as a source of carbon and energy. The first step in the utilization of acetate is its activation into acetyl coenzyme A (Ac-CoA). Once Ac-CoA is made, it is used to synthesize lipids, can enter the tricarboxylic acid cycle to generate precursors of amino acids, or can be oxidized to generate reducing power via diverse pathways (9, 15, 26, 57).

In *B. subtilis*, like in many other prokaryotes, acetate is excreted into the environment until the compound from which it is derived (e.g., glucose) is depleted. At that point, the acetate switch (55) is flipped, and acetate recapture is initiated (1, 10, 35).

Two pathways are central to acetate catabolism in prokaryotes. Both of these pathways activate acetate into Ac-CoA, but they do it via different intermediates and in response to different concentrations of acetate in the environment. The pathway relevant to this work is the one illustrated in reactions 1 and 2 below, both of which are catalyzed by the Ac-CoA synthetase (AcsA) EC 6.2.1.1 (49).



The second pathway is comprised of the acetate kinase (AckA; EC 2.7.2.1) and the phosphotransacetylase (Pta; EC 2.3.1.8) enzymes (6). AckA catalyzes reaction 3, while Pta catalyzes reaction 4.



Although both pathways are reversible, hydrolysis of pyrophosphate generated by reaction 1 prevents the AcsA from converting Ac-CoA to acetate. The role of these pathways is to carefully maintain a physiological balance of Ac-CoA and free CoA (13, 14, 55).

In *B. subtilis*, the Pta/AckA pathway appears to be a carbon overflow pathway used for the excretion of acetate (21). In contrast, the AcsA enzyme is preferentially used to capture acetate from the environment (53). The interplay of these acetate utilization pathways is carefully controlled at the transcription level by the global regulators CcpA and CodY. The latter represses *acsA* while activating *ackA* expression (34, 43, 44, 46).

Previous work from our laboratory identified some of the functions required for the posttranslational control of the *B. subtilis* AcsA enzyme in vitro. Enzymes encoded by the *acuA* and *acuC* genes were shown to inactivate and reactivate the Ac-CoA-forming activity of AcsA (17). In *B. subtilis*, the *acuA* and *acuC* loci are adjacent to the *acsA* gene. A gene of unknown function, *acuB*, is located between *acuA* and *acuC* (16, 17). In this report, we provide evidence that the AcsA enzyme of *B. subtilis* is posttranslationally regulated in vivo, and we identify an additional protein deacetylase enzyme involved in the control of the activity of AcsA.

At present, expression of *acuABC* genes appears to be responsive to conditions relevant to spore germination and out-

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growth and during anaerobic respiration to nitrate (8). The data showing AcuA involvement in spore germination are relevant because they suggest that posttranslational modification is a mechanism used by the cell to control central metabolic functions other than AcsA.

The regulation of expression of the *acuABC* genes is complicated by the fact that the operon is under the control of the global regulatory protein CcpA, which, as mentioned above, also regulates the expression of the *acsA* gene. The tight regulation at the transcriptional level of the *acsA* gene and the effective posttranslational control of AcsA activity reflect on the importance of maintaining CoA homeostasis in this bacterium.

The posttranslational control system that regulates the AcsA enzyme of *B. subtilis* appears to be a variation of the one first described for *Salmonella enterica*, whose Acs enzyme is under acetylation control (48). In *S. enterica*, a protein acetyltransferase (Pat) enzyme homologous to the Gcn-5 protein of *Saccharomyces cerevisiae* (30, 50) uses Ac-CoA to acetylate a lysyl residue in the active site of Acs, which stops the first step of Ac-CoA formation (48) (reaction 1). The cognate deacetylase in *S. enterica* is a class III protein deacetylase that uses NAD<sup>+</sup> as a substrate (not as a coenzyme), yielding *O*-acetyl-ADP-ribose (*O*-AADPR) and nicotinamide as by-products (25, 29, 39, 40, 51). Class III histone deacetylases are called sirtuins because they are homologous to the founding member of this family of proteins, the SIR2 protein of *Saccharomyces cerevisiae* (5, 36). In the gram-negative enterobacterium *S. enterica*, sirtuin-type deacetylase activity is associated with the CobB protein (45, 48, 52).

In this paper, we provide in vitro and in vivo evidence that the YhdZ protein of *B. subtilis* has sirtuin-like activity; that is, YhdZ has NAD<sup>+</sup>-dependent protein deacetylase activity. To avoid confusion generated by the use of a gene name that reflects unknown function (i.e., *yhdZ*), hereinafter we refer to *yhdZ* as *srtN* (for sirtuin). We present evidence that indicates that the posttranslational modification system controlling the *B. subtilis* AcsA enzyme is more complex than the one in *S. enterica*, because two deacetylases appear to be involved in the deacetylation of AcsA<sup>Ac</sup>.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** Table 1 lists genotypes of strains of *S. enterica* serovar Typhimurium LT2 and *B. subtilis* SMY used in these studies. *S. enterica* strains were grown on no-carbon essential medium (2), supplemented with MgSO<sub>4</sub> (1 mM) and L-methionine (0.5 mM). *B. subtilis* strains were grown on a modified Spizizen's minimal medium (47). The latter contained distilled water (90 ml), 10× *Bacillus* salts (10 ml; stock [wt/100 ml of water], sodium citrate [1 g], KH<sub>2</sub>PO<sub>4</sub> [6 g], K<sub>2</sub>HPO<sub>4</sub> [14 g], ammonium sulfate [2 g], MgSO<sub>4</sub> [17 mg]), filter-sterilized trace elements (100 μl; stock [wt/100 ml of water], CaCl<sub>2</sub> [5.5 mg], FeCl<sub>2</sub> [14 mg], MnCl<sub>2</sub> · 4H<sub>2</sub>O [1 mg], ZnCl<sub>2</sub> [2 mg], CuCl<sub>2</sub> · 2H<sub>2</sub>O [0.4 mg], CoCl<sub>2</sub> · 6H<sub>2</sub>O [0.6 mg], Na<sub>2</sub>MoO<sub>4</sub> [0.6 mg], 1 M HCl [200 μl]), and glutamic acid (2 mg). A low concentration of acetate (10 mM for *S. enterica*, 30 mM for *B. subtilis*) or propionate (30 mM) was used as the sole carbon and energy source for cells grown in minimal medium. L-(+)-Arabinose (250 μM) or isopropyl-β-D-thiogalactoside (IPTG; 400 μM) was used to induce expression of plasmid-borne genes. Lysogenic broth (LB) was used as rich medium (3, 4). When used, antibiotics were present in the medium at the following concentrations: ampicillin, 100 μg/ml; erythromycin, 1 μg/ml; chloramphenicol, 5 μg/ml; spectinomycin, 100 μg/ml; and kanamycin, 10 μg/ml. All chemicals were purchased from Sigma.

**Phage P22-mediated transduction of *S. enterica* genes.** Lysates of the high-transducing strain HT105/1 *int-201* (41, 42) of bacteriophage P22 were used as

donors to move chromosomal markers into specific genetic backgrounds. Protocols for P22-mediated transductions are described elsewhere (11).

**Mobilization of plasmids.** Plasmids were introduced into *S. enterica* or *B. subtilis* strains by transformation or electroporation (23, 33, 37).

**Construction of *B. subtilis* strains.** We replaced the *acuC* gene of *B. subtilis* strain SMY with a spectinomycin resistance cassette from plasmid pDG1726 (*Bacillus* Genetic Stock Center) using described PCR protocols (54). We used the same method to inactivate the *srtN* gene with either a spectinomycin or an erythromycin resistance cassette from plasmids pDG1726 or pDG646, respectively. The *acuA* gene was inactivated by the insertion of a kanamycin resistance gene, which was cut from plasmid pDG783 using restriction enzyme EcoRI (Promega). The fragment containing the kanamycin resistance marker was purified, blunted with DNA polymerase I Klenow fragments (Fermentas), and repurified with a commercially available kit (Qiagen). Plasmid pACUA2 was cut with enzyme SacI, which was cut only once and within the *acuA* gene. The linearized plasmid was purified and blunted with DNA polymerase I Klenow fragments. The kanamycin resistance marker was ligated into the linearized pACUA2 plasmid and transformed into *E. coli* strain DH5α, selecting for kanamycin resistance on rich medium containing kanamycin. Plasmid DNA was recovered from the *kan*<sup>+</sup> transformants, and the *acuA::kan*<sup>+</sup> construct was amplified and purified. The resulting 2.1-kb PCR fragment was transformed into strain JE9089 (*B. subtilis* *acuC1::spc*<sup>+</sup> *srtN5::erm*<sup>+</sup>), selecting for kanamycin resistance. The presence of the *acuA::kan*<sup>+</sup> insertion in the chromosome of the resulting strain was confirmed by PCR analysis.

**PCR.** All amplifications used *TripleMaster* polymerase (Eppendorf) and were performed using an Eppendorf Mastercycler gradient PCR thermocycler (Brinkmann Instruments). Primers were purchased from Integrated DNA Technologies (see Table S1 in the supplemental material).

**Cloning of *B. subtilis* genes.** Template DNA was obtained from *B. subtilis* strain SMY. For complementation studies with *S. enterica*, the *srtN* gene was cloned into the EcoRI and HindIII sites of plasmid pBAD30 (22).

For complementation studies with *B. subtilis*, the *srtN* gene was amplified from genomic DNA purified from *B. subtilis* SMY with primers that had NotI and SalI restriction sites at the 5' and 3' ends of the gene, respectively. The *srtN* PCR product, which did not include its promoter, was purified and cut with NotI and SalI enzymes. The *B. subtilis*⇌*E. coli* shuttle plasmid pHP13 (a gift from A. L. Sonenshein) was cut with the HindIII and SalI enzymes and purified. The low-level, constitutive promoter P<sub>spac</sub> drove expression of the *srtN* gene. P<sub>spac</sub> was cut from plasmid pMUTIN4, using HindIII and SalI restriction enzymes. We sequentially cloned P<sub>spac</sub> and *srtN*<sup>+</sup> into plasmid pHP13, resulting in plasmid pSRTN3 (*srtN*<sup>+</sup>). For a scheme of the construction of plasmid pSRTN3, see Fig. S3 in the supplemental material. For the purpose of protein purification, the *srtN*<sup>+</sup> gene was cloned into the NdeI and PstI sites of plasmid pTYB12 (New England BioLabs).

**Protein overproduction and isolation.** (i) **SrtN.** SrtN protein was overproduced in *E. coli* strain ER2566 harboring plasmid pSRTN2 (pTYB12 *srtN*<sup>+</sup>). Cells were grown with shaking (180 rpm) at 30°C to an optical density at 650 nm of 0.4, at which point IPTG was added to a final concentration of 400 μM to induce expression of the *srtN*<sup>+</sup> gene. After induction, cultures were incubated with shaking (180 rpm) at 18°C for 16 h. Cells were broken using a French press (Spectronic Unicam) under 1.26 kPa of pressure. Cell debris was removed by centrifugation at 12,000 × g for 45 min at 4°C in an Avanti J-251 centrifuge (Beckman-Coulter); clarified, crude cell extract was filtered using a 45-μm filter syringe. Crude cell extract was loaded onto a 5-ml chitin affinity chromatography column (New England BioLabs), which was developed per the manufacturer's instructions. Purified SrtN protein was dialyzed against HEPES buffer (0.05 M, pH 7.5) containing KCl (0.1 M) and glycerol (25%, vol/vol).

(ii) **AcsA and GST-AcuA.** AcsA and glutathione *S*-transferase (GST)-AcuA proteins were purified using previously described protocols (16, 17). All proteins were drop frozen in liquid nitrogen and stored at -80°C until used.

(iii) **PncA.** Nicotinamidase (PncA) was purified as described previously (18) and was a gift from A. Tucker.

**Protein purity analysis.** Protein purity was assessed using Fotodyne's FOTO/Eclipse electronic documentation and analysis system, including software packages FOTO/Analyst PC Image v5.0 and TotalLab 1D gel analysis v2003 from NonLinear Dynamics, Ltd. This analysis was performed using sodium dodecyl sulfate (SDS)-polyacrylamide gels (27) stained with Coomassie blue (38).

**Determination of the molecular mass of the SrtN protein.** The molecular mass of the SrtN protein was determined using fast protein liquid chromatography on a Superdex-200 column (Amersham) equilibrated and developed with 50 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl at a flow rate of 0.5 ml/min. A standard curve was generated using proteins of known molecular mass. For this purpose, we used gel filtration standard mixture (Bio-Rad Labo-

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype	Source or reference <sup>a</sup>
<i>S. enterica</i> serovar Typhimurium strains		
LT2	Wild type	
ER2566	F <sup>-</sup> λ <sup>-</sup> <i>fhuA2</i> [ <i>lon</i> ] <i>ompT lacZ</i> ::T7 gene1 <i>gal sulA11</i> Δ( <i>mcrC-mrr</i> )114::IS10 R( <i>mcr-73</i> ::miniTn10-Tet <sup>s</sup> )2 R( <i>zgb-210</i> ::Tn10) (Tet <sup>s</sup> ) <i>endA1</i> [ <i>dcm</i> ]	New England BioLabs
LT2 derivative		
TR6583	<i>metE205 ara-9</i>	K. Sanderson via J. Roth
TR6583 derivatives		
JE4718	<i>cobB1176</i> ::Tn10d( <i>tet</i> <sup>+</sup> ) <i>pta102</i> ::MudI1734	
JE7724	<i>cobB1176</i> ::Tn10d( <i>tet</i> <sup>+</sup> ) <i>pta102</i> ::MudI1734/pBAD30	
JE7725	<i>cobB1176</i> ::Tn10d( <i>tet</i> <sup>+</sup> ) <i>pta102</i> ::MudI1734/pCOBB8	
JE7726	<i>cobB1176</i> ::Tn10d( <i>tet</i> <sup>+</sup> ) <i>pta102</i> ::MudI1734/pSRTN1	
<i>B. subtilis</i> strain		
JE8607	SMY (prototroph)	
SMY derivatives		
JE8610	<i>acuC1</i> :: <i>spc</i> <sup>+</sup>	
JE8611	<i>srtN4</i> :: <i>spc</i> <sup>+</sup>	
JE9089	<i>acuC1</i> :: <i>spc</i> <sup>+</sup> <i>srtN5</i> :: <i>erm</i> <sup>+</sup>	
JE11111	<i>acuC1</i> :: <i>spc</i> <sup>+</sup> <i>srtN5</i> :: <i>erm</i> <sup>+</sup> /pSRTN3	
JE11281	<i>acuC1</i> :: <i>spc</i> <sup>+</sup> <i>srtN5</i> :: <i>erm</i> <sup>+</sup> <i>acuA2</i> :: <i>kan</i> <sup>+</sup>	
Plasmids		
pBAD30	Expression vector, P <sub>araBAD</sub> <i>bla</i> <sup>+</sup>	11
pHP13	<i>E. coli</i> - <i>B. subtilis</i> shuttle vector, <i>cat</i> <sup>+</sup>	A. L. Sonenshein
pDG646	Antibiotic cassette harboring vector for <i>B. subtilis</i> , replicates in <i>E. coli</i> , <i>bla</i> <sup>+</sup> in <i>E. coli</i> , <i>erm</i> <sup>+</sup> in <i>B. subtilis</i>	BGSC
pDG783	Antibiotic cassette harboring vector for <i>B. subtilis</i> , replicates in <i>E. coli</i> , <i>bla</i> <sup>+</sup> in <i>E. coli</i> , <i>kan</i> <sup>+</sup> in <i>B. subtilis</i>	BGSC
pDG1726	Antibiotic cassette harboring vector for <i>B. subtilis</i> , replicates in <i>E. coli</i> , <i>bla</i> <sup>+</sup> in <i>E. coli</i> , <i>spc</i> <sup>+</sup> in <i>B. subtilis</i>	BGSC
pMUTIN4	P <sub>spac</sub> promoter harboring vector for <i>B. subtilis</i> , replicates in <i>E. coli</i> , <i>bla</i> <sup>+</sup>	BGSC
pTYB12	Purification vector, N-terminal self-cleavable intein tag, <i>bla</i> <sup>+</sup>	New England BioLabs
pACUA2	<i>B. subtilis</i> <i>acuA</i> <sup>+</sup> cloned into pTYB12 <i>bla</i> <sup>+</sup>	
pCOBB8	<i>S. enterica</i> <i>cobB</i> <sup>+</sup> cloned into pBAD30	
pSRTN1	<i>B. subtilis</i> <i>srtN</i> <sup>+</sup> cloned into pBAD30 <i>bla</i> <sup>+</sup>	
pSRTN2	<i>B. subtilis</i> <i>srtN</i> <sup>+</sup> cloned into pTYB12 <i>bla</i> <sup>+</sup>	
pSRTN3	<i>B. subtilis</i> <i>srtN</i> <sup>+</sup> cloned into pHP13	
	Under the control of the P <sub>spac</sub> promoter, <i>cat</i> <sup>+</sup>	

<sup>a</sup> Unless otherwise stated, strains were constructed during the course of this work. BGSC, *Bacillus* Genetic Stock Center.

ratories). The compensation volume was 0.5 ml, and the empty loop volume was 0.2 ml. Protein elution was monitored at 215 and 280 nm over a period of 60 min. A plot of molecular mass (kilodaltons) versus retention time (min) was generated using the above-mentioned protein standards. The standard curve was used to determine the approximate molecular mass and oligomeric state of active SrtN enzyme.

**SrtN (sirtuin)-dependent protein deacetylation assay.** The GST-AcuA-dependent acetylation of the AcsA reaction was performed with a volume of 100 μl under described conditions (17). [<sup>14</sup>C,C-1]Ac-CoA (Moravak Biochemicals) was present in the reaction mixture at a specific radioactivity of 47 mCi/mmol; the concentration of Ac-CoA in the reaction mixture was 20 μM. After a 2-h incubation period at 37°C, GST-AcuA protein was removed from the reaction using 20 μl of a 50% (vol/vol) slurry of GST·Mag beads (Novagen) equilibrated with HEPES buffer (0.05 M, pH 7.5) containing tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 200 μM). Incubation and removal of the magnetic beads was performed per the manufacturer's instructions. After removal of the beads, 185 pmol of purified SrtN deacetylase and 1 mM NAD<sup>+</sup> were added to the sample. In some experiments, PncA nicotinamidase (128 pmol) was added to the reaction mixture to relieve SrtN inhibition by nicotinamide. Samples were incubated at 37°C for 2 h, and the reaction was stopped by precipitation with trichloroacetic

acid to a final concentration to 5% (wt/vol). Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and phosphorimaging analysis.

## RESULTS AND DISCUSSION

Although control of AcsA activity by acetylation has been reported, the supporting data were obtained in vitro or by heterologous complementation of *S. enterica* strains by *B. subtilis* genes provided in *trans*, but experiments with the appropriate *B. subtilis* strains were not performed (17).

Here, we present results from experiments performed with strains of *B. subtilis* with an altered AcsA posttranslational modification system. The data indicate that the AcuA protein is the acetyltransferase that modifies and inactivates AcsA, and AcuC is one of two deacetylases that reactivates AcsA<sup>Ac</sup>. The other deacetylase is encoded by *yhdZ*, which we show in vivo and in vitro to be a class III NAD<sup>+</sup>-dependent sirtuin deacetylase.

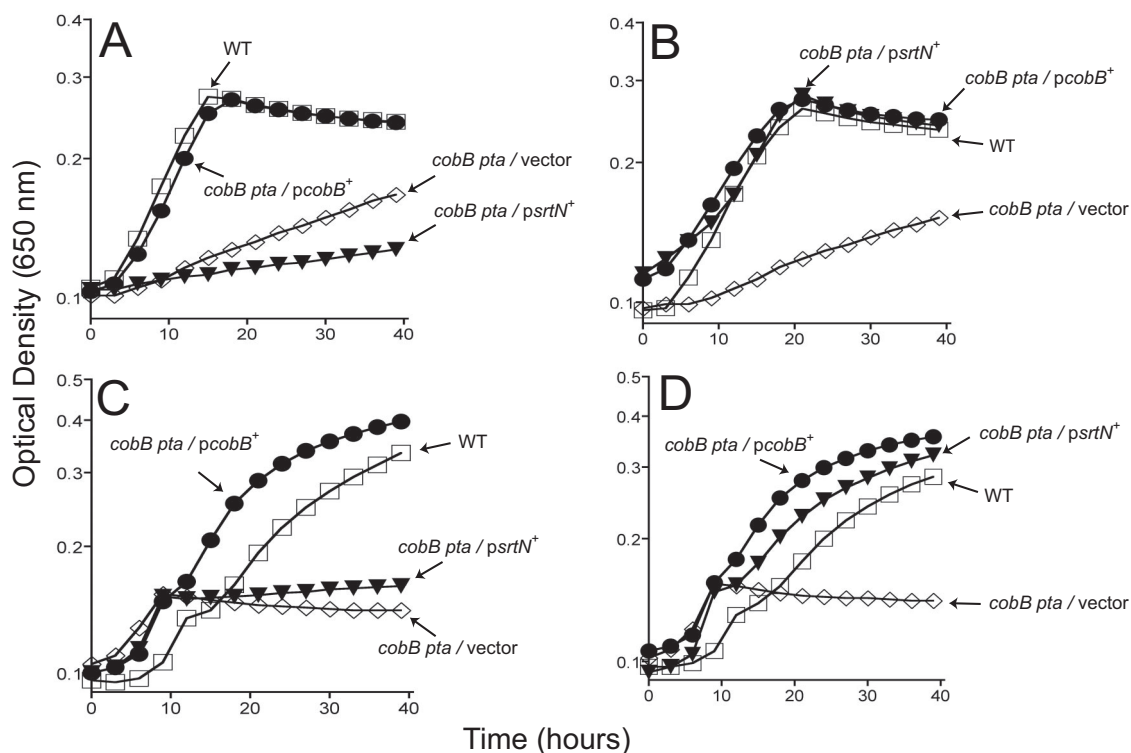


FIG. 1. *B. subtilis* SrtN compensates for the lack of CobB during growth of a *S. enterica cobB* strain under low-acetate or -propionate conditions. Growth behavior of *B. subtilis* strains in 10 mM acetate (A and B) or 30 mM propionate (C and D). In panels B and D, the expression of plasmid-borne *cobB<sup>+</sup>* and *srtN<sup>+</sup>* was by the inclusion L<sup>+</sup>-arabinose (250  $\mu$ M) in the medium. Open squares, JE4175 (wild type); open diamonds, JE7724 (*cobB pta*/vector); filled circles, JE7725 (*cobB pta*/pCOBBB8 *cobB<sup>+</sup>*); filled inverted triangles, JE7726 (*cobB pta*/pSRTN1 *srtN<sup>+</sup>*). On the graph, *pcobB<sup>+</sup>* is pCOBB8, and *psrtN<sup>+</sup>* is pSRTN1. The growth experiments were performed at least three times, and each experiment was done in triplicate. The data presented here have error bars showing variability; however, in most cases, they are smaller than the symbol. WT, wild type.

lase. To reflect the sirtuin protein deacetylase activity associated with the YhdZ protein, we propose a change in gene nomenclature, from *yhdZ* to *srtN*.

**SrtN compensates for the lack of CobB sirtuin during growth of a *S. enterica cobB* strain under low-acetate or -propionate conditions.** To obtain evidence that SrtN was a bona fide sirtuin, we performed heterologous complementation experiments using *S. enterica* strain JE4718 (*cobB pta*). We introduced plasmid pSRTN1 (*srtN<sup>+</sup>*) into strain JE4718, resulting in strain JE7726. Inactivation of the *pta* gene in JE7726 was needed to force the conversion of acetate or propionate to Ac-CoA or propionyl-CoA via the Acs enzyme. Because the lack of *cobB* function blocks deacetylation (hence activation) of Acs<sup>Ac</sup>, growth of strain JE7726 on acetate or propionate would occur only if *B. subtilis* SrtN deacetylated Acs<sup>Ac</sup>.

Arabinose-induced expression of the *B. subtilis srtN<sup>+</sup>* gene restored growth of strain JE7726 on either acetate (Fig. 1B) or propionate (Fig. 1D). A *S. enterica cobB* strain, which could not deacetylate Acs<sup>Ac</sup> (48), exhibited a partial defect in growth on acetate (Fig. 1A and B). As a positive control, we introduced plasmid pCOBB8 (*cobB<sup>+</sup>*) into strain JE4718. As expected, the expression of *cobB<sup>+</sup>* restored growth on acetate in the presence or absence of arabinose (Fig. 1B). Expression of the *B. subtilis srtN<sup>+</sup>* gene restored growth of the *S. enterica cobB* strain (Fig. 1A and B). But, unlike *cobB<sup>+</sup>*, higher expression of *srtN<sup>+</sup>* was needed to support growth of the *S. enterica cobB* strain on acetate (Fig. 1C and D). It is unclear whether

the need for induction of *srtN<sup>+</sup>* reflects the stability of SrtN in *S. enterica* or differences in the interactions of SrtN with the *S. enterica* Acs protein. Regardless of the reason, the data indicate that SrtN can substitute for CobB during growth of a *S. enterica cobB* strain under low-acetate or -propionate conditions, supporting the conclusion that the *B. subtilis* SrtN protein is a sirtuin.

**NAD<sup>+</sup>-dependent protein deacetylase (sirtuin) activity associated with SrtN.** The end-to-end homology shared by the SrtN and the *S. enterica* CobB proteins (see Fig. S1 in the supplemental material) also suggested SrtN was a sirtuin. We tested this idea in vitro using purified SrtN (>95% homogeneous), AcsA (>95% homogeneous), and GST-AcuA (>95% homogeneous) proteins. Radiolabeled AcsA<sup>Ac</sup> protein was generated by incubation with AcuA and [<sup>14</sup>C,C-1]Ac-CoA. SrtN was added to the reaction mixture after removal of GST-AcuA, and the amount of label associated with AcsA was quantified by phosphorimaging. The data showed that the SrtN enzyme deacetylated about 50% of AcsA<sup>Ac</sup> protein in the mixture and that deacetylation required NAD<sup>+</sup> as a cosubstrate (Fig. 2, lane 3), as do other sirtuins (24, 28, 45). In addition, SrtN was inhibited by nicotinamide, another hallmark of sirtuins (Fig. 2, lane 4) (12, 19, 32); nicotinamide is the by-product of the SrtN reaction. The addition of nicotinamide (PncA) increased SrtN activity to almost 72% (Fig. 2, lane 5), consistent with SrtN being a sirtuin. This amount of deacetylation was comparable to that of the AcuC protein

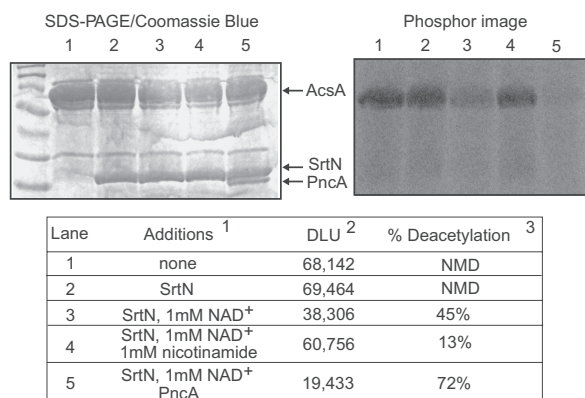


FIG. 2. SrtN-dependent deacetylation of *B. subtilis* AcsA<sup>Ac</sup>. The acetyltransferase reaction was performed as described in Materials and Methods. The acetyltransferase reaction mixture contained HEPES buffer (0.05 M, pH 7.5) and TCEP (200  $\mu$ M) as a disulfide bond-reducing agent, GST-AcuA (3  $\mu$ g [130 pmol]), and AcsA, (10  $\mu$ g [170 pmol]). Amounts of protein added per 100- $\mu$ l reaction mixture were as follows: for SrtN, 5  $\mu$ g (185 pmol); and for PncA, 3  $\mu$ g (128 pmol). SrtN-dependent AcsA<sup>Ac</sup> deacetylation was assessed after removal of GST-AcuA from the reaction mixture. Deacetylation reaction mixtures were incubated at 37°C for 2 h and were stopped by precipitation with trichloroacetic acid. Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and quantification of AcsA<sup>Ac</sup> in was performed by phosphorimaging analysis. Footnotes: 1, concentration in the reaction mixture; 2, digital light units (DLU) reflecting the amount of AcsA<sup>Ac</sup>; 3, normalized to DLU measured in the absence of SrtN. NMD, no measurable deacetylation.

deacetylase (17). Finally, like other sirtuins, *B. subtilis* SrtN was active as a monomer (see Fig. S2 in the supplemental material) (31, 58).

**In *B. subtilis*, SrtN and AcuC protein deacetylases are required for efficient growth of *B. subtilis* under low-acetate conditions.** Analysis of the functionality of AcsA in vivo necessitates the use of low concentrations of acetate (30 mM). Higher concentrations of acetate would be metabolized by the Pta/AckA pathway, obscuring the analysis of AcsA activity as a function of its acetylation state. Attempts to eliminate the contribution of the Pta/Ack pathway were not successful. Although we were able to construct *pta acuC* and *pta srtN* strains, all efforts to construct the *pta acuC srtN* strain failed, suggesting that *pta acuC srtN* is a synthetic lethal combination.

A disadvantage to using 30 mM acetate as the sole source of carbon and energy is that the cell yield is low, and differences in the growth rate are not dramatic. However, with the appropriate controls, one can discern the effect caused by the absence of the AcuC and SrtN functions. We found that the use of viable counts was the best approach to analyze the roles of AcuA, AcuC, and SrtN function during growth under low-acetate conditions. Relative to the wild-type strain, a strain lacking *acuC* displayed a reproducible and statistically significant growth defect under low-acetate conditions (Fig. 3). The observed growth defect was substantially more pronounced in the *acuC srtN* strain (Fig. 3), suggesting that the *srtN* and *acuC* functions were needed to maintain AcsA as active (i.e., deacetylated). If this idea were correct, one would expect the growth behavior of the *srtN acuC* strain to be similar to that of the *acsA* strain, and it was (Fig. 3). Expression of the *srtN*<sup>+</sup> in

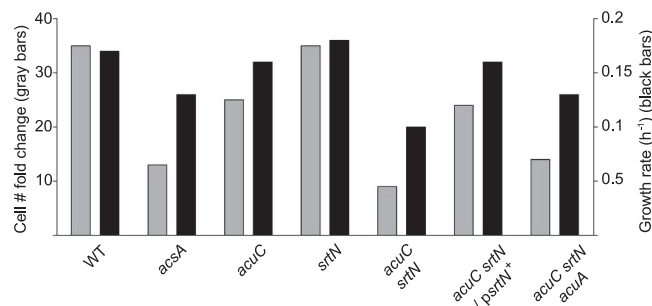


FIG. 3. Growth of *Bacillus subtilis* strains on 30 mM acetate. The effects of the acetylation/deacetylation mutations on cell growth were measured by the change in cell number and the growth rate. For a statistical analysis of these growth data, please see Table S2 in the supplemental material. WT, wild type.

*trans* under the control of the low-level constitutive P<sub>spac</sub> promoter restored growth of the *srtN acuC* strain on acetate to the level seen in the *acuC* strain. As expected, the rate of growth of the *srtN acuC/psrtN*<sup>+</sup> culture was similar to that of the *srtN acuC*<sup>+</sup> culture and slower than that of the wild-type strain (Fig. 3). Based on these data, we concluded that efficient growth of *B. subtilis* on acetate required both AcuC and SrtN deacetylase activities.

We also predicted that inactivation of *acuA* in the *acuC srtN* strain would result in an unmodified (active) AcsA protein and that growth would improve. As predicted, growth of the *acuC srtN acuA* strain on acetate did improve, but it did not match that of the wild-type strain. The fact that inactivation of *acuA* did not restore wild-type growth of the *acuA acuC srtN* strain under low-acetate conditions suggests that the absence of AcuC and/or SrtN may affect the function of yet-to-be-identified proteins.

In summary, the posttranslational modification system that controls the activity of the Ac-CoA synthetase enzyme in the gram-positive bacterium *B. subtilis* is more complex than the previously studied one in the gram-negative bacterium *S. enterica*. Notable differences between the two systems include the following. (i) The genes encoding the protein acetyltransferase and one deacetylase enzymes may be cotranscribed in *B. subtilis*, but not in *S. enterica*. (ii) The *B. subtilis* *acuABC* genes are under transcriptional control of a global regulator responsive to the quality of the carbon source available to the cell (20, 21). At present, the transcriptional control of the *S. enterica* *pat* and *cobB* genes have not been reported. (iii) Unlike *S. enterica*, *B. subtilis* uses two different types of deacetylases to control AcsA activity, one whose activity depends on NAD<sup>+</sup> (SrtN) and another whose activity does not (AcuC) (Fig. 4). Bioinformatics searches for other protein deacetylases in *B. subtilis* failed to identify any orthologues of *acuC* and *srtN*, suggesting that if there are other protein deacetylases in this bacterium, they may belong to a new class of this family of enzymes (56).

It is not obvious why *B. subtilis* would require redundancy in the deacetylases that control AcsA activity. The obvious difference between AcuC and SrtN functions is that the latter requires NAD<sup>+</sup>, while the former does not use NAD<sup>+</sup> as cosubstrate (17). We speculate that the involvement of AcuC and SrtN in AcsA<sup>Ac</sup> reactivation may occur in response to

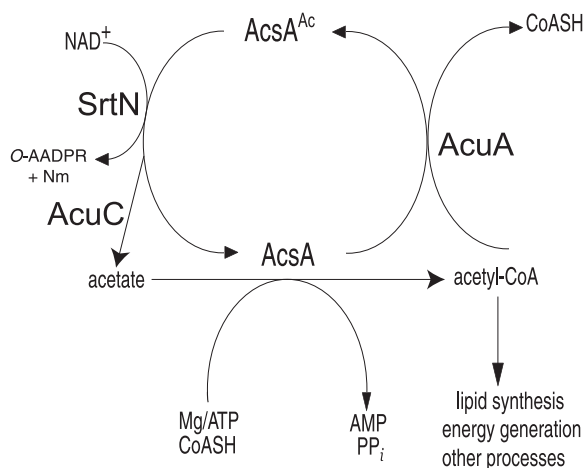


FIG. 4. Schematic of the control of AcsA by the acetylation/deacetylation system of *B. subtilis*. While the data show that AcuA is the only acetyltransferase that can acetylate (deactivate) AcsA, the deacetylation (re-activation) step can be catalyzed by the SrtN or AcuC deacetylases. The physiological conditions under which each of the deacetylases is preferentially active are presently unknown. CoASH, free CoA.

different indicators of the energy charge of the cell. AcuC-dependent activation of Acs<sup>Ac</sup> may be triggered in response to a low Ac-CoA:CoA ratio, allowing the cell to replenish Ac-CoA to levels needed for survival. The involvement of SrtN may be triggered when the NAD<sup>+</sup>:NADH ratio becomes high, a signal of high energy charge in the cell. Under such physiological conditions (high-energy and low-Ac-CoA conditions), the cell would need to meet the demand for Ac-CoA; thus, it would use SrtN to activate AcsA<sup>Ac</sup>. These ideas are currently under investigation.

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