Short-Chain Fatty Acid Activation by Acyl-Coenzyme A Synthetases Requires SIR2 Protein Function in *Salmonella enterica* and *Saccharomyces cerevisiae*

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ABSTRACT

SIR2 proteins have NAD^+-dependent histone deacetylase activity, but no metabolic role has been assigned to any of these proteins. In *Salmonella enterica*, SIR2 function was required for activity of the acetyl-CoA synthetase (Acs) enzyme. A greater than two orders of magnitude increase in the specific activity of Acs enzyme synthesized by a sirtuin-deficient strain was measured after treatment with homogeneous *S. enterica* SIR2 protein. Human SIR2A and yeast SIR2 proteins restored growth of SIR2-deficient *S. enterica* on acetate and propionate, suggesting that eukaryotic cells may also use SIR2 proteins to control the synthesis of acetyl-CoA by the level of acetylation of acetyl-CoA synthetases. Consistent with this idea, growth of a quintuple *sir2 hst1 hst2 hst3 hst4* mutant strain of the yeast *Saccharomyces cerevisiae* on acetate or propionate was severely impaired. The data suggest that the Hst3 and Hst4 proteins are the most important for allowing growth on these short-chain fatty acids.

SHORT-CHAIN fatty acids (SCFAs) such as acetate and propionate are used as sources of carbon and energy by prokaryotes occupying diverse habitats such as soil, where acetate and propionate are the most abundant fatty acids (Buckel 1999), or the gastrointestinal tract of humans, where the concentration of acetate and propionate can reach high levels (Cummings *et al.* 1987). All catabolic pathways for acetate and propionate require these SCFAs to be activated into their corresponding SCFAcyl-CoA forms before they can be converted into metabolites that can enter central metabolism. Acetyl-CoA feeds directly into the TCA cycle, whereas propionyl-CoA can be catabolized via a number of different pathways that convert it into pyruvate, acetate, or succinyl-CoA, which then enter the TCA cycle (Horswill and Escalante-Semerena 1997).

In enteric bacteria such as *Escherichia coli* and *Salmonella enterica*, acetate is activated into acetyl-CoA via either one of two pathways (Figure 1). The first pathway requires the involvement of the acetate kinase (AckA, EC 2.7.2.1) and phosphotransacetylase (Pta, EC 2.3.1.8) enzymes. In these bacteria AckA and Pta are responsible for the synthesis of acetyl-CoA when acetate is present in high concentrations in the environment (≥30 mm acetate). This pathway is considered to be the low-affinity pathway for acetate activation. The second pathway for the activation of acetate requires the activity of the ATP-dependent acetate:CoA ligase (AMP forming, EC 6.2.1.1; *i.e.*, acetyl-CoA synthetase) encoded by the *acs* gene. Acetyl-CoA synthetase (Acs) is required when the concentration of acetate in the environment is low (≤10 mm acetate); thus this pathway is considered to be the high-affinity pathway for acetate activation. In *S. enterica* propionate can be activated to propionyl-CoA by the ATP-dependent propionate:CoA ligase (AMP forming, EC 6.2.1.17; *i.e.,* propionyl-CoA synthetase) encoded by the *prpE* gene of the *prpBCDE* operon (Horswill and Escalante-Semerena 1997, 1999a, 2001, 2002). The *prpBCDE* operon of this bacterium encodes functions needed for the catabolism of propionate via the 2-methylcitric acid cycle (Horswill and Escalante-Semerena 1999b, 2001). In addition, *S. enterica* has two distinct propionate kinases (PduW, TdcD), but the genes encoding these enzymes (*pduW*, *tdcD*) are part of the *p*ropane*d*iol *u*tilization (*pduABCDEGHJKLMNOPQSTUVWX*) and *t*hreonine *d*e*c*arboxylation (*tdcBCDEG*) operons whose expression is induced only under specific growth conditions (Hesslinger *et al.* 1998; Bobik *et al.* 1999; S. Palacios and J. C. Escalante-Semerena, unpublished results). Hence, under conditions where the *pduW* and *tdcD* genes are not expressed, propionate activation to propionyl-CoA occurs only via the high-affinity propionyl-CoA synthetase-dependent pathway (Horswill and Escalante-Semerena 1999a).

S. enterica mutant strains that carry a wild-type *prpBCDE* operon and are unable to grow on propionate as carbon and energy source have been isolated. One of these mutant strains is of particular interest because its inability to grow on propionate is due to the inactivation of the *cobB* gene, which encodes a homolog of the SIR2 family of eukaryotic regulatory proteins (*i.e.*, sirtuins; Tsang and Escalante-Semerena 1998). Sirtu-

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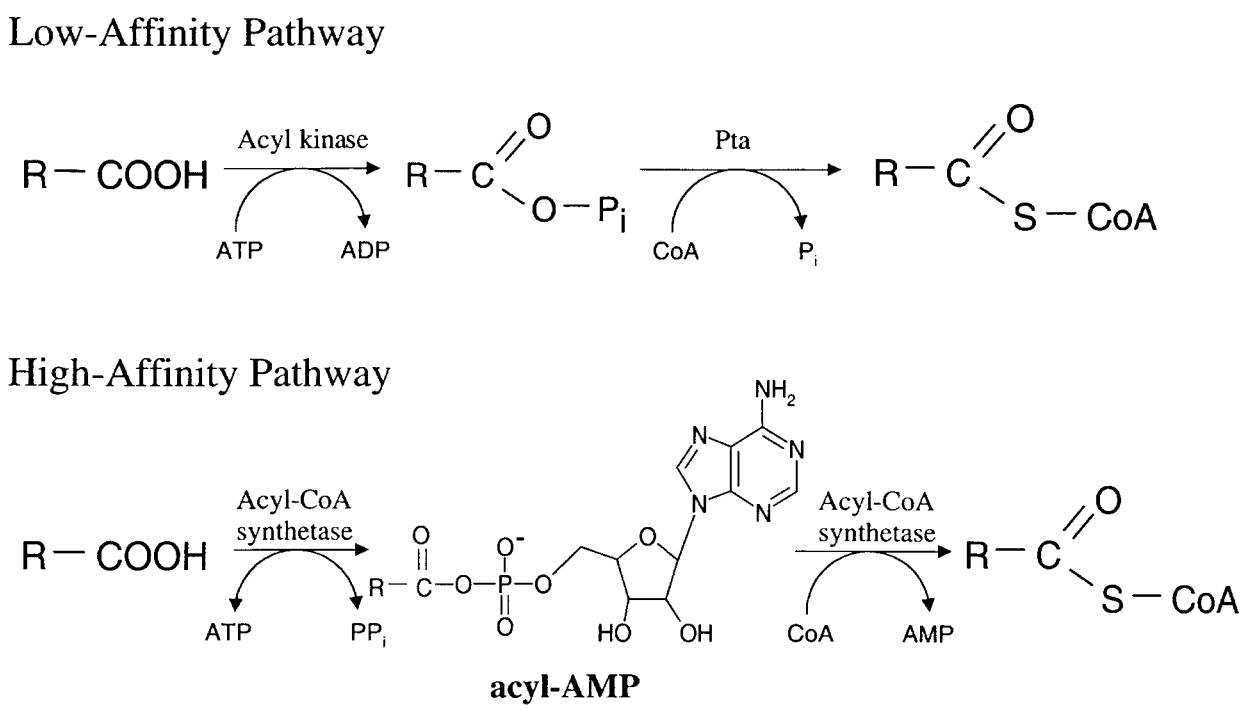

FIGURE 1.—Pathways for SCFAcyl-CoA synthesis in *S. enterica*. In the low-affinity pathway, acetate kinase (AckA, EC 2.7.2.1) catalyzes the synthesis of acetyl-phosphate, two propionate kinases (encoded by *pduW* and *tdcD*) catalyze the synthesis of propionyl-phosphate, and phosphotransacetylase (Pta, EC 2.3.1.8) converts acyl-phosphate to acyl-CoA. The high-affinity pathway of acyl-CoA synthesis involves AMP-forming acyl-CoA synthetases.

ins from eukaryotes and prokaryotes are Zn-containing proteins with NAD^+-dependent histone deacetylase activity that are implicated in the process of gene silencing and cell aging (FREY 1999; TANNY *et al.* 1999; IMAI *et al.* 2000; LANDRY *et al.* 2000a,b; SMITH *et al.* 2000; TANNER *et al.* 2000; TANNY and MOAZED 2000; MCVEY *et al.* 2001; MIN *et al.* 2001; SAUVE *et al.* 2001; TISSENBAUM and GUARENTE 2001). The sirtuin protein of the archaeon *Sulfolobus sulfataricus* was recently shown to regulate the affinity of the major chromatin protein for DNA in this prokaryote by modulating the level of acetylation of this protein (BELL *et al.* 2002).

In this article, we report results from experiments aimed at identifying a role for sirtuins in metabolism, with the ultimate goal of learning about how metabolic processes may affect cell aging. The data obtained indicate that in *S. enterica* CobB sirtuin function is required for the activation of the short-chain fatty acids acetate and propionate to their corresponding acyl-CoA derivatives by acyl-CoA synthetases (BROWN *et al.* 1977; KUMARI *et al.* 1995; HORSWILL and ESCALANTE-SEMERENA 1999a). Both human SIR2A and the yeast SIR2 proteins restored growth of sirtuin-deficient strains of *S. enterica* on acetate and propionate, suggesting that this newly identified metabolic role of sirtuins may be widely distributed or that the specificity of sirtuins for their substrates is not very high. The yeast *Saccharomyces cerevisiae* encodes five sirtuins in its genome. These include the founding member of the family, *SIR2* itself, a closely related paralog, *HST1*, *HST2*, a more distantly related paralog, and a pair of genes, *HST3* and *HST4*, that are the most distantly related to *SIR2* but are closely related to each other. Previous studies show that *hst3 hst4* mutants show a variety of synthetic phenotypes, including slow growth, temperature sensitivity, and a variety of cell cycle and chromosome instability phenotypes (BRACHMANN *et al.* 1995). In support of the idea that the conclusions reached by studying *S. enterica* might have phylogenetically wider implications, a strain of *S. cerevisiae* lacking all five sirtuin functions was shown to grow poorly on acetate as carbon and energy source. Further analysis of these phenotypes suggested that the Hst3 and Hst4 sirtuins are most important for allowing growth on these SCFAs.

MATERIALS AND METHODS

Bacterial and yeast strains, media, chemicals, and growth conditions: All bacterial strains used in this study were derivatives of *S. enterica* serovar Typhimurium LT2. The genotypes of bacterial and yeast strains and plasmids used are listed in Table 1. *S. enterica* strains were grown on minimal medium (BERKOWITZ *et al.* 1968) supplemented with $MgSO_4$ (1 mM), L-methionine (0.5 mM), and a trace minerals solution (BALCH and WOLFE 1976). Luria-Bertani broth (LB) was the rich medium used to grow *S. enterica* strains. Growth curves were performed in 96-well microtiter dishes (Becton-Dickinson, Cockeysville, MD) using a computer-controlled SpectraMAX PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA), with the incubation chamber set at 37°. A 2-μl sample of an overnight culture of *S. enterica* was used to inoculate 198 μl of

TABLE 1

Strains and plasmid list

Strain or plasmid	Relevant genotype	Source or reference[a]
	S. enterica strains[b]	
LT2	Wild type	Laboratory collection
Derivatives of LT2		
TR6583	*metE205 ara-9*	K. Sanderson via J. Roth
Derivatives of TR6583		
JE1208	*ack101::*MudJ[c]	Laboratory collection
JE1209	*pta102::*MudJ	Laboratory collection
JE2445	*cobB1176::*Tn*10*d(Tc)	Laboratory collection
JE4175	TR6583/pBAD30 *bla*$^+$	Laboratory collection
JE4313	Δ*1231*(*acs*) *prpE213::kan*$^+$	Laboratory collection
JE4314	Δ*1231*(*acs*) *prpE213::kan*$^+$/pBAD30	Laboratory collection
JE4349	Δ*299*(*hisG-cob*)/pGP1-2/pCOBB6	Laboratory collection
JE4597	*pta209::*Tn*10*	
JE4718	*cobB1176::*Tn*10*d(Tc) *pta102::*MudJ	
JE4872	*cobB1176::*Tn*10*d(Tc) *pta102::*MudJ/pBAD30	
JE5318	*cobB1176::*Tn*10*d(Tc) *pta102::*MudJ/pCOBB8	
JE5944	*cobB1176::*Tn*10*d(Tc)/pTDCD1	
JE6175	*cobB1176::*Tn*10*d(Tc) *pta102::*MudJ/pTDCD1	
JE6227	Δ*1231*(*acs*) *prpE213::kan*$^+$/pTDCD1	
JE6290	*pta209::*Tn*10*Δ*1231*(*acs*) *prpE213::kan*$^+$	
JE6531	*cobB1176::*Tn*10*d(Tc)/pACK3	
JE6533	*cobB1176::*Tn*10*d(Tc) *pta102::*MudJ/pACK3	
JE6534	Δ*1231*(*acs*) *prpE213::kan*$^+$/pACK3	
JE6557	*cobB1176::*Tn*10*d(Tc)/pGEX-*huSIR2A*	
JE6558	*cobB1176::*Tn*10*d(Tc)/pGEX-*SIR2*	
JE6669	Δ*1231*(*acs*) *ack101::*MudJ	
JE6670	*cobB1176::*Tn*10*d(Tc) *ack101::*MudJ	
	S. cerevisiae strains	
Derivatives of YPH499[d]		
YPH499	*MAT***a** *SIR2*$^+$ *HST*$^+$	Sikorski and Hieter (1989)
YCB173	*MAT***a** *sir2*Δ*1::URA3*	Brachmann *et al.* (1995)
YCB515	*MAT***a** *hst1*Δ*2::LEU2*	Brachmann *et al.* (1995)
YCB405	*MAT***a** *hst3*Δ*3::HIS3*	Brachmann *et al.* (1995)
YCB523	*MAT***a** *hst4*Δ*1::URA3*	Brachmann *et al.* (1995)
YCB235	*MAT***a** *sir2*Δ*1::URA3 hst1*Δ*2::LEU2*	Brachmann *et al.* (1995)
YCB538	*MAT***a** *hst3*Δ*3::HIS3 hst4*Δ*1::URA3*	Brachmann *et al.* (1995)
YCB547	*MAT*α *hst3*Δ*3::HIS3 hst4*Δ*1::URA3*	Brachmann *et al.* (1995)
YCB498	*MAT***a** *sir2*Δ*1::URA3 hst1*Δ*2::LEU2 hst2*Δ*1::TRP1 hst3*Δ*3::HIS3 hst4*Δ*1::URA3*	Brachmann *et al.* (1995)
Derivatives of FY2[e]		
YCB617	*MAT***a** *SIR2*$^+$ *HST*$^+$ *leu2::TRP1*	Brachmann *et al.* (1995)
YCB426	*MAT***a** *sir2*Δ*2::TRP1*	Brachmann *et al.* (1995)
YCB423	*MAT***a** *hst1*Δ*3::TRP1*	Brachmann *et al.* (1995)
YCB1097	*MAT***a** *hst2*Δ*2::TRP1*	Brachmann *et al.* (1995)
YCB470	*MAT***a** *hst3*Δ*3::TRP1*	Brachmann *et al.* (1995)
YCB575	*MAT***a** *hst4*Δ*1::TRP1*	Brachmann *et al.* (1995)
	Plasmids	
pBAD30	Expression vector, P_{araBAD} *bla*$^+$	Guzman *et al.* (1995)
pGEM-T	Cloning vector, *bla*$^+$	Promega
pCOBB8	*cobB*$^+$ cloned into pBAD30, *bla*$^+$	
pTDCD1	*tdcD*$^+$ cloned into pBAD18s, *bla*$^+$	Laboratory collection
pACK3	*ackA*$^+$ cloned into pBAD30, *bla*$^+$	
pCAR325	Human SIR2A (SIRT2) cloned into pGEX-4T3	C. Brachmann and J. D. Boeke, unpublished data
pGEX-SIR2	Yeast *SIR2* cloned into pGEX	D. Moazed

[a] Lack of a reference or source indicates the strain was constructed during the course of this work.
[b] All *S. enterica* strains used in this study were derivatives of *S. enterica* serovar Typhimurium LT2.
[c] MudJ is an abbreviation of MudI1734 (Castilho *et al.* 1984).
[d] Other markers common to this yeast strain background used: *ura3-52 his3*Δ*200 leu2*Δ*1 trp1*Δ*63 ade2-101 lys2-801.*
[e] Other markers used: *ura3-52 his3*Δ*200 leu2*Δ*1 trp1*Δ*63 lys2*Δ*202.*

freshly prepared minimal medium in each well, supplemented with the appropriate carbon source at the indicated concentrations. Data points were collected every 5 min, with shaking for 240 sec between readings. Expression of genes under the control of the P_{araBAD} promoter was induced by including L-(+)-arabinose at a final concentration of 200 μM for propionate growth and 500 μM for acetate growth. Ampicillin was used at 100 μg/ml. All chemicals were purchased from Sigma (St. Louis), unless otherwise stated. [1-^{14}C]Acetate (sp. act., 51 mCi/mmol) and [1-^{14}C]propionate (sp. act., 55 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). Yeast media were based on yeast peptone (YP) base, which is 10 g/liter yeast extract and 20 g/liter Bacto-peptone, and were supplemented with 0.32 g/liter L-tryptophan and 0.184 g/liter adenine hemisulfate (to suppress red pigment formation in *ade2* mutants). YPD medium was prepared as described (Rose *et al.* 1990). YP0 medium contained no added carbon/energy source; YPD contained (111 mM or 2% w/v) dextrose; YPAc contained NaAcetate (244 mM or 2% w/v); YPPro contained Na propionate (50 mM) and glycerol (1 mM); YPGE contained glycerol (218 mM or 2% w/v) and ethanol (2% v/v).

Construction of the quintuple mutant yeast strain: A series of *hst* disruption mutations was generated in the YPH499/500 background (Sikorski and Hieter 1989) or the FY2 background (Winston *et al.* 1995). In the former background, the following alleles were constructed: *hst1Δ2::LEU2, hst2Δ2::TRP1, hst3Δ3::HIS3, hst4Δ1::URA3*, and *sir2Δ1::URA3*. In the latter background, *hst1Δ3::TRP1, hst2Δ1::TRP1, hst3Δ3::TRP1, hst4Δ1::TRP1*, and *sir2Δ2::TRP1* were created. Details of alleles structures and strain construction have been published (Brachmann 1996).

Plasmid constructions: *Construction of plasmid pACK3:* The *S. enterica ackA* gene was PCR amplified from the chromosome, using the forward primer 5′ GCTACGCTCTATGGCTCA 3′ and the reverse primer 5′ GAAATCAGGCAGTCAGAC 3′. The sequence used to obtain these primers was made available from the *S. typhimurium* genome sequencing project at Washington University at St. Louis (http://genome.wustl.edu/gsc/projects/S.typhimurium). The resulting 1.2-kb fragment containing *ackA* was A-tailed and ligated into pGEM-T (Promega, Madison, WI), according to manufacturer's instructions. The resulting plasmid contained the *ackA* gene in the orientation for expression from P_{lacZ}. This intermediate vector was digested with *Sac*I and *Sph*I, and the 1.3-kb fragment containing the *ackA* gene was gel purified away from the linearized vector. This insert was ligated into the arabinose-inducible vector pBAD30 (Guzman *et al.* 1995), which was digested with the same enzymes. The resulting 6.2-kb plasmid was named pACK3.

Construction of plasmid pCOBB8: The *cobB* gene was amplified from the *S. enterica* chromosome using the forward primer 5′ TTACATCTTACCGACTAATC 3′ and the reverse primer 5′ CGTAACGTGAAATGTAGGC 3′. An 898-bp fragment was A-tailed and ligated into vector pGEM-T, according to manufacturer's instructions. This intermediate vector contained the *cobB* gene in the orientation for expression from the P_{lacZ} promoter. This construct was digested with *Sac*I and *Sph*I enzymes, and the 968-bp $cobB^+$ fragment was ligated into vector pBAD30 cut with the same enzymes. The resulting 5.9-kb plasmid was named pCOBB8.

Construction of plasmid pCAR325 (pGEX4T3-huSIR2A): Human *Sir2A* cDNA was obtained from an EST clone (GenBank accession no. T66100). The sequence was determined (S. Devine, C. B. Brachmann and J. D. Boeke, unpublished data) and the insert was released by digestion with *Nco*I and filling in with Klenow fragment; following phenol extraction *Not*I was added. This insert was inserted into expression vector pGEX4T-3 (Pharmacia) between the *Sma*I and *Not*I sites, generating an in-frame glutathione S-transferase fusion. This protein has been expressed and purified and has NAD^+-dependent histone deacetylase activity (Smith *et al.* 2000).

Phage P22 transductions: All transductional crosses were performed as previously described (Davis *et al.* 1980) with phage P22 HT105/1 *int*-210 (Schmieger 1971; Schmieger and Bakhaus 1973). Transductants were freed of phage as described (Chan *et al.* 1972).

Determination of the rates of accumulation of propionate and acetate: A 50-μl sample of an overnight culture of the appropriate *S. enterica* strain grown in LB at 37° was used to inoculate 5 ml of minimal medium containing succinate (30 mM) as the carbon source (to allow growth of sirtuin mutant strains) and 15 mM of either acetate or propionate. These cultures were grown at 37° to an optical density (OD_{600}) of 0.7. At this point, 1.5 ml of culture was harvested by centrifugation with an IEC Centra-M centrifuge (International Equipment, Needham Heights, MA) at 13,000 × *g* for 2 min. The supernatant was decanted, and the cell pellet was washed twice with minimal medium lacking a carbon source. After the second wash, the cell pellet was resuspended in 300 μl of NCE supplemented with $MgSO_4$ and L-methionine. These suspensions were incubated at 37° in a Tropi-Cooler variable temperature block (Boekel Scientific, Feasterville, PA), until the start of the assay. The assay was started by the addition of 100 μl of prewarmed cell suspension to 2 ml of minimal medium in 13 × 100 mm test tubes, also prewarmed to 37°. Mixing was achieved by vortexing and tubes were placed in a shaking water bath set to 37° for 7 min, after which radiolabeled [1-^{14}C]acetate or [1-^{14}C]propionate was added to a final concentration of 200 μM. The specific activities of radiolabeled acetate or propionate in the medium were 9.2 mCi/mmol and 9.9 mCi/mmol, respectively. Samples (100 μl each) of the mixture were withdrawn at 1-min intervals over 10 min, filtered through 0.45-μm filter discs (Pall Life Sciences, Ann Arbor, MI) under vacuum and washed with 10 ml of ice-cold 50 mM sodium phosphate buffer, pH 7.0, containing 10 mM of nonradioactive acetate or propionate. The filter discs were then placed into 6 ml of Scinti-Safe scintillation fluid (Fisher Scientific, Pittsburgh) and counted in a Packard Tri-Carb 2100TR scintillation counter (Packard Instrument, Downers Grove, IL) for 1 min. Time zero time points included all components of the assay, except for the addition of cells. The remaining 200 μl of the cell suspension was used to determine protein concentration.

Determination of acetyl-CoA synthetase activity: Five-ml overnight cultures of the appropriate *S. enterica* strains grown in LB at 37° were subcultured into 500 ml of minimal medium containing 30 mM succinate as a carbon and energy source. These cultures were allowed to grow overnight with shaking at 37°. The cells were harvested by centrifugation at 9000 × *g* for 15 min with a Sorvall RC-5B refrigerated centrifuge (Dupont Instruments, Wilmington, DE) fitted with a GSA rotor. The cell pellets were resuspended in 10 ml 50 mM HEPES buffer, pH 7.5, containing 200 μM Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride (Pierce Chemical, Rockford, IL) as a reducing agent. Cells were broken in a French press (Aminco). Crude extracts were collected and immediately dialyzed in SnakeSkin 10,000 MWCO dialysis tubing (Pierce) against 500 ml of the original resuspension buffer at 4°. Each extract was allowed to dialyze for a minimum of 3 hr, with buffer changes each hour. Dialyzed cell-free extracts were collected, and protein concentration was determined (Bradford 1976). Reaction mixtures (final volume, 100 μl) contained 50 mM HEPES buffer, pH 7.5, 200 μM TCEP, 236 μM radiolabeled [1-^{14}C]acetate (specific activity, 8.1 mCi/mmol), 5 mM coenzyme A, and 5 mM Mg/ATP. Reactions were started by the addition of cell-free extract (100 μg of protein). Some reaction mixtures also contained 10 μg of purified *S. enterica* CobB sirtuin. When added, NAD^+ was present at a final concentration of 5 mM. Reaction mixtures were incubated at 37° for 1 hr, stopped by the addition of 20 μl of 1 M formic acid, and filtered through 0.45-μm Spin-X centrifuge tube filters

(Corning, Corning, NY) in an IEC Centra-M centrifuge (International Equipment Company) at 13,000 × *g* for 5 min. Components of the reaction mixtures were resolved by thin layer chromatography (TLC) on Whatman PE SIL G/UV silica gel TLC (Whatman, Maidstone, Kent, England). Five microliters from each mixture was spotted onto a 20 × 20-cm plate, allowed to dry, and developed with a chloroform:methanol (3:2) solvent system. Acetyl-coenzyme A was retained at the origin under these conditions. Free, underivatized acetate was clearly resolved from acetyl-CoA (*Rf* = 0.89). The amount of label retained at the origin was determined by scintillation counting using a Packard Tri-Carb 2100TR scintillation counter for 1 min. Background level was determined with reactions that lacked protein extract, but contained all other components of the reaction.

Protein determination: Protein concentration in samples was determined using the Bradford Bio-Rad protein assay protocol (Bio-Rad Laboratories, Hercules, CA) with BSA as standard, according to manufacturer's instructions.

Purification of the CobB sirtuin: *Salmonella enterica* strain JE4349 was used to overexpress *cobB* as described (Tsang and Escalante-Semerena 1998). Cells were harvested by centrifugation (10,415 × *g*) at 4° for 10 min with a Sorvall GSA rotor and RC-5B refrigerated centrifuge (DuPont). Cells were disrupted using a French pressure cell (Spectronic, Rochester, NY). For this purpose cell pellets were resuspended in 35 ml of 50 mm Tris-HCl buffer, pH 7.5 (at 4°), 1 mm EDTA, 200 μm phenylmethylsulfonyl fluoride, and 5 mm dithiothreitol (DTT) and broken with two passes in the French press at 1.034 × 10^8 kPa. Cell debris was discarded by centrifugation at 43,140 × *g* for 45 min at 4° using a Sorvall SS34 rotor (DuPont). CobB sirtuin was precipitated out of the resulting clarified cell-free extract (31 ml) with ammonium sulfate (41% saturation) on ice. Precipitated proteins were allowed to stand on ice for 10 min and were collected by centrifugation at 11,951 × *g* in a Sorvall SS34 rotor. The protein pellet was solubilized with 10 ml of 50 mm Tris-HCl buffer, pH 7.5 (at 4°), 1 mm EDTA, and 1 mm DTT. Purified CobB sirtuin was dialyzed overnight at 4° against 1 liter of the same buffer, loaded onto a 20-ml (1.5 × 11 cm) fast-flow DEAE-650M ToyoPearl anion exchange resin (Rhom & Haas) equilibrated with 50 mm Tris-HCl buffer, pH 7.5 (at 4°), 1 mm EDTA, and 1 mm DTT at a rate of 100 ml/hr. After loading the protein on the column, the latter was washed with 30 ml of the equilibration buffer, followed by a 200-ml gradient from zero to 0.25 m NaCl in the same equilibration buffer. Five-ml fractions were collected, and their protein contents were analyzed by SDS-PAGE (Laemmli 1970) and Coomassie blue staining (Sasse 1991). CobB sirtuin eluted between 0.1 to 0.2 m NaCl. Fractions containing CobB sirtuin were pooled and concentrated using Centricon concentrators (Millipore, Bedford, MA) to a final volume of 11 ml. Concentrated fractions were dialyzed overnight at 4° against 1 liter of equilibration buffer. Dialyzed CobB sirtuin was loaded onto a 10-ml (1.5 × 5.5 cm) Cibacron Blue 3GA (Sigma) column equilibrated with the same equilibration buffer at 15 ml/hr. After loading, the column was washed with 40 ml of the equilibration buffer, 3-ml fractions were collected, and CobB sirtuin was eluted in the wash step. Fractions containing the bulk of the CobB sirtuin were pooled and concentrated to ~2 ml. The concentrated protein was saved at −90° in 50% glycerol, 25 mm Tris-HCl buffer at pH 7.5 (at 4°), 1 mm EDTA, and 10 mm DTT.

RESULTS

Eukaryotic sirtuins compensate for the lack of CobB sirtuin function during growth of *S. enterica* on propionate: Previous work showed that sirtuin-deficient strains of *S. enterica* grow very poorly on propionate as carbon and energy source, but the precise role of the sirtuin in propionate catabolism remained unclear (Tsang and Escalante-Semerena 1996). To investigate whether eukaryotic sirtuins could compensate for the lack of sirtuin activity in *S. enterica cobB* mutants during growth on propionate, plasmids carrying cDNA clones of either the human SIR2A gene (pCAR325-*huSIR2A*) or the *S. cerevisiae SIR2* gene (pGEX-*SIR2*) were introduced into strain JE2445 [*cobB1176::*Tn*10*d(Tc)]. Figure 2 shows representative growth behavior data of the *cobB* mutant carrying the human sirtuin *in trans*. Low-level expression of the human SIR2A protein restored growth of the *cobB* mutant on propionate to almost wild-type rates (Figure 2, solid diamonds *vs.* solid squares). In contrast, increased expression of the human sirtuin resulted in the complete inhibition of growth (Figure 2, open diamonds). Similar observations were made when yeast Sir2p was overexpressed in yeast (Holmes *et al.* 1997). The mechanism of growth inhibition in *S. cerevisiae* was proposed to be defects in chromosome segregation resulting from altered histone acetylation. The reason for the observed growth inhibition in *S. enterica* is unclear. Expression of the *S. cerevisiae* gene failed to compensate

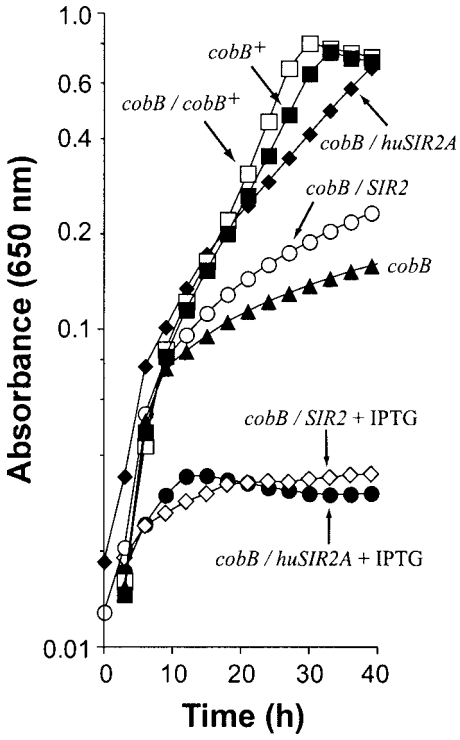

Figure 2.—Eukaryotic sirtuins restore growth of *S. enterica* sirtuin mutants on propionate. The concentration of propionate in the medium was 30 mm. Additional components of the medium are described in materials and methods. JE4175 (*cobB*$^+$/pBAD30), solid squares; strain JE4872 (*cobB*/pBAD30), solid triangles; JE5318 (*cobB*/pCOBB8), open squares; JE6557 (*cobB*/pGEX-huSIR2A), solid diamonds; JE6558 (*cobB*/pGEX-SIR2), open circles. Growth response upon induction of the pGEX plasmids by 50 μm isopropyl thiogalactoside is represented by open diamonds (strain JE6557).

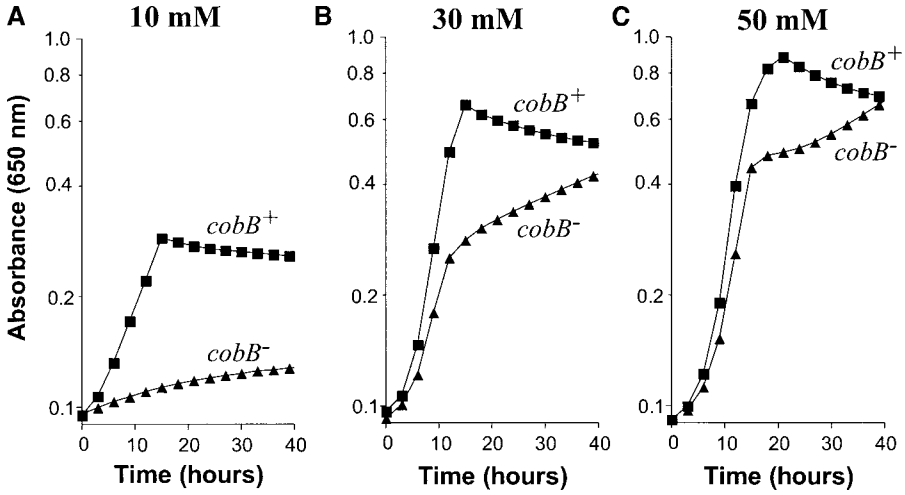

FIGURE 3.—Growth of sirtuin-proficient and sirtuin-deficient strains of *S. enterica* on acetate. The concentration of acetate in the medium was as indicated. The growth behavior of strains TR6583 (*cobB*$^+$) and JE2445 [*cobB1176*::Tn*10*d (Tc)] is shown by squares and triangles, respectively.

for the lack of CobB sirtuin as efficiently as the human gene did (Figure 2, open circles), but the observed improvement measured in the presence of the yeast sirtuin was significant and reproducible. These results indicated that the eukaryotic sirtuins were able to perform whatever role the CobB sirtuin plays in propionate catabolism in *S. enterica*.

Sirtuin-deficient strains of *S. enterica* grow poorly on low levels of acetate: As shown in Figure 3, sirtuin mutants were also unable to use acetate as carbon and energy source. At a low concentration (10 mM), acetate failed to support growth of the sirtuin mutant (Figure 3A, triangles) in spite of the fact that the strain carried in its genome a wild-type allele of the *acs* gene encoding the high-affinity acetyl-CoA synthetase enzyme. The wild-type strain grew well under these conditions. In contrast, high concentrations of acetate in the medium (*i.e.*, 30–50 mM) greatly improved growth of the sirtuin mutant (Figure 3, B and C). In medium containing 30 mM acetate the doubling time of the sirtuin mutant strain (doubling time, 9 hr, Figure 3B, triangles) almost matched the rate of the wild-type strain (doubling time, 7 hr, Figure 3B, squares). When the concentration of acetate was increased to 50 mM, the doubling times of the *cobB*$^-$ and *cobB*$^+$ strains were almost identical (Figure 3C, doubling time, 5.7 hr, *cobB*$^+$, squares *vs.* doubling time, 6 hr, *cobB*$^-$, triangles) consistent with the idea that sirtuin function was needed for activation of acetate by the high-affinity Acs enzyme, but not for the activation of acetate by the low-affinity acetate kinase (AckA)/Pta enzyme system.

Growth of *S. enterica* sirtuin mutants on propionate or acetate is restored upon overexpression of an acetyl or propionyl kinase enzyme: On the basis of the results presented above, it was predicted that expression of an acyl kinase would bypass the need for sirtuin function during growth on acetate or propionate. To test this idea, the propionate kinase enzyme encoded by *tdcD* and the acetate kinase enzyme encoded by *ackA* were cloned separately on a vector containing an arabinose-inducible promoter. Data in Table 2 show that overexpression of *ackA* allowed the sirtuin-deficient strain to reach a cell density similar to that of the wild-type strain (Table 2, column E, line 3 *vs.* 5) at approximately the

TABLE 2

Acyl kinase-dependent growth rates of *cobB* mutant strains

	A. Strain	B. Relevant genotype[a]	C. Doubling time on propionate[a] (hr)	D. Doubling time on acetate[a] (hr)	E. Maximum optical density (650 nm) and time (hr) required to reach it during growth on acetate
1	JE4175	*cobB*$^+$/pBAD30	10	6	0.62 at 15 hr
2	JE4872	*cobB*$^-$/pBAD30	45	6	0.35 at 27 hr
3	JE5318	*cobB*$^-$/pCOBB8 (*cobB*$^+$)	11	8	0.54 at 27 hr
4	JE5944	*cobB*$^-$/pTDCD1 (*tdcD*$^+$)	10	8	0.57 at 27 hr
5	JE6531	*cobB*$^-$/pACK3 (*ackA*$^+$)	17	6	0.62 at 27 hr
6	JE6175	*cobB*$^-$ *pta*$^-$/pTDCD1 (*tdcD*$^+$)	NG	NG	NG
7	JE6533	*cobB*$^-$ *pta*$^-$/pACK3 (*ackA*$^+$)	NG	NG	NG

NG, no growth.

[a] All strains were grown with 30 mM acetate or propionate as the sole carbon and energy source. Expression of *tdcD*$^+$, *ackA*$^+$, and *cobB*$^+$ from the indicated plasmids required 200 μM arabinose for propionate growth and 500 μM arabinose for growth on acetate.

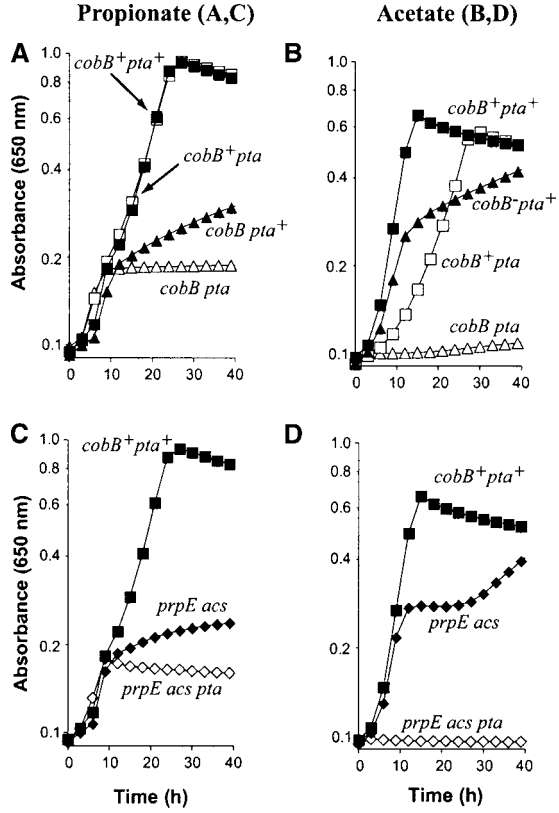

FIGURE 4.—Evidence for sirtuin-dependent and sirtuin-independent activation of acetate and propionate. Growth behavior of strains on 30 mM propionate (A and C) and 30 mM acetate (B and D). Solid squares, TR6583 (*cobB*$^+$ *prpE*$^+$ *acs*$^+$ *pta*$^+$); solid triangles, JE2445 (*cobB*$^-$ *prpE*$^+$ *acs*$^+$ *pta*$^+$); open squares, JE4597 (*cobB*$^+$ *prpE*$^+$ *acs*$^+$ *pta*); solid triangles, JE4718 (*cobB prpE*$^+$ *acs*$^+$ *pta*); solid diamonds, JE4313 (*cobB*$^+$ *prpE acs pta*$^+$); and open diamonds, JE6290 (*cobB*$^+$ *prpE acs pta*).

same rate on acetate medium (Table 2, column D, line 3 *vs.* 5). Growth of the sirtuin mutant on propionate was restored by either a *cobB*$^+$ or a *tdcD*$^+$ allele *in trans* (Table 2, columns D and E, line 3 *vs.* 4). These data indicated that the synthesis of propionyl-phosphate by overproduced TdcD enzyme could bypass the need for sirtuin function. AckA only partially substituted for TdcD during growth on propionate (Table 2, column C, line 3 *vs.* 5). This was evidence that AckA could synthesize propionyl-phosphate *in vivo*. Similarly, the TdcD enzyme partially substituted for AckA during growth on acetate (Table 2, columns D and E, line 4 *vs.* 5).

Phosphotransacetylase enzyme activity is required for propionate kinase-dependent growth of an *S. enterica* sirtuin mutant on propionate: The above results suggested that the low-affinity system of acyl-CoA synthesis was responsible for sirtuin-independent synthesis of acetyl- and propionyl-CoA. To investigate this possibility, the *pta* gene was inactivated in several genetic backgrounds, and growth of the mutant strains on acetate or propionate was assessed. As shown in Figure 4, the sirtuin mutant grew poorly on propionate as carbon and energy source, but this growth behavior was reproducible. The rate of growth of the sirtuin mutant was sixfold slower (Figure 4A, solid triangles, doubling time, 36 hr) than that of the wild-type strain (Figure 4A, solid squares, doubling time, 6 hr). The slow, but reproducible growth of the sirtuin-deficient strain on propionate was completely eliminated upon inactivation of the *pta* gene (Figure 4A, open triangles). Overexpression of the propionate kinase encoded by the *tdcD*$^+$ allele (plasmid pTDCD1 (P_{araBAD}-*tdcD*$^+$) failed to restore growth of strain JE4718 (*cobB*$^-$ *pta*$^-$) on propionate (data not shown), a result consistent with the sirtuin-independent pathway of acyl-CoA synthesis being the low-affinity acyl-CoA synthesis pathway. Strain JE4597 (*cobB*$^+$ *pta*$^-$) grew as well as strain TR6583 (*cobB*$^+$ *pta*$^+$) on propionate (Figure 4A, open squares *vs.* solid squares), indicating that Pta function was not required for the sirtuin-dependent pathway. Similar results were obtained when the experiment was repeated with acetate as carbon and energy source (Figure 4B).

Sirtuin-dependent growth of *S. enterica* on acetate or propionate requires acetyl- or propionyl-CoA synthetase activity: It was important to determine whether sirtuin

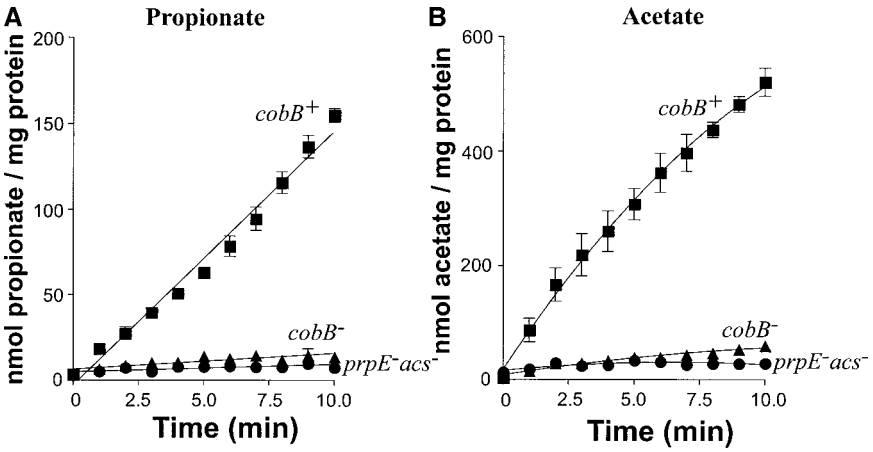

FIGURE 5.—Kinetics of SCFA accumulation in wild-type and mutant strains. (A) Propionate accumulation. (B) Acetate accumulation. The kinetics of accumulation of SCFAs by the sirtuin-proficient, acyl-CoA synthetase-proficient (*cobB*$^+$ *prpE*$^+$ *acs*$^+$) strain is shown by squares; the kinetics for the sirtuin mutant (*cobB*) strain is shown by triangles, and the kinetics for the acyl-CoA synthethase double mutant (*prpE acs*) strain is shown by circles.

function was required for the synthesis of acyl-CoA via the low-affinity acyl kinase/phosphotransacetylase system or via the high-affinity acyl-CoA synthetase-dependent pathway (Figure 1). Toward this end, genes encoding acyl-CoA synthetases capable of synthesizing propionyl-CoA (*i.e.*, *prpE*, *acs*; Horswill and Escalante-Semerena 1999a) were inactivated in a strain carrying the wild-type *cobB*$^+$ allele. As seen in Figure 4C, strain JE4313 (*cobB*$^+$ *Δ1231acs prpE213::kan*$^+$) grew very poorly on propionate (solid diamonds; doubling time, 50 hr), but this growth was reproducible. Inactivation of the *pta* gene in strain JE4313 completely blocked growth on propionate (Figure 4C, open diamonds). Similar results were obtained when acetate was used as the sole source of carbon and energy. Unlike growth on propionate, however, growth of the acyl-CoA sythetase double mutant (*prpE acs*) on acetate was biphasic (Figure 4D, solid diamonds). The meaning of this behavior is unclear. These results indicated that sirtuin function was part of the high-affinity, acyl-CoA synthetase-dependent pathway of acyl-CoA synthesis.

In *S. enterica*, the lack of sirtuin or acyl-CoA synthetase (Acs/PrpE) activities result in a drastic decrease in the intracellular level of propionate or acetate: The intracellular level of acetate and propionate was measured to determine if the observed lack of growth on these SCFAs was due to insufficient levels of substrate for the acyl-CoA synthetases. As seen in Figure 5A, the rate of intracellular accumulation of propionate in the sirtuin mutant was ~16-fold slower (0.93 ± 0.22 nmol of propionate accumulated per milligram of protein per minute) than the rate measured in the sirtuin-proficient strain (14.84 ± 0.50 nmol of propionate accumulated per milligram of protein per minute; Figure 5A, squares *vs.* triangles). An even more pronounced effect in the rate of propionate accumulation was measured in the strain lacking acyl-CoA synthetase activities (Acs, PrpE; 0.43 ± 0.09 nmol of propionate accumulated per milligram of protein per min; Figure 5A, squares *vs.* circles). Similar results were obtained when acetate accumulation was assessed (Figure 5B).

Sirtuin function is required for growth of *S. cerevisiae* on acetate or propionate: The yeast *S. cerevisiae* genome contains five sirtuins, *SIR2* and *HST1–4*. We examined whether defects in SCFA metabolism were evident by examining the growth properties of yeast cells bearing mutations in *SIR2* or its paralogues, *HST1*, *HST2*, *HST3*, and *HST4*. The growth of these mutants was analyzed on rich (YP) medium containing various carbon and energy sources, including acetate and propionate. No defects were noted in any of the single mutants (Figure 6A). However, quintuple *sir2 hst1 hst2 hst3 hst4* mutant strains had significant growth defects on the SCFA-containing plates (Figure 6B, bottom). These growth defects became worse as the concentration of SCFA increased (not shown). These defects appeared to be specific to SCFAs because these growth defects were not observed

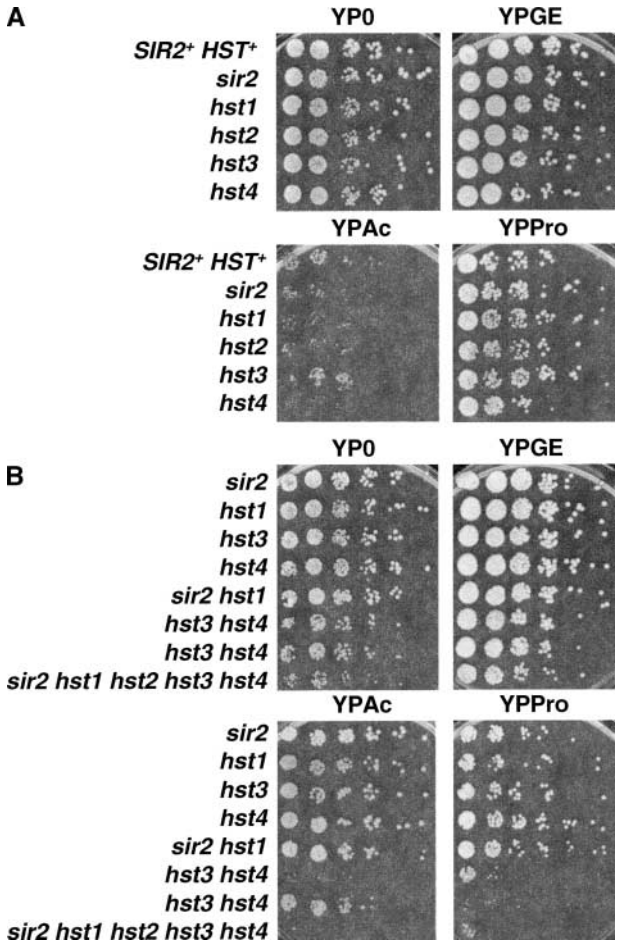

Figure 6.—Yeast sirtuin mutants grow poorly on SCFA-containing media. (A) Single *sir2* and *hst* mutants grow normally on SCFA-containing media. Yeast cells grown on YPD plates were resuspended in water, adjusted to OD_{600} of 0.1, and serially diluted fivefold in a 96-well tray. Three microliters of each suspension was spotted onto the yeast extract peptone media plates containing the following carbon sources: YP0, no additional carbon source; YPGE, glycerol and ethanol; YPAc, acetate; and YPPro, propionate (see materials and methods). The plates were incubated for 1 week in a humidified chamber at 30°. Strains spotted were (top to bottom): YCB617 (*SIR2*$^+$ *HST*$^+$), YCB426 (*sir2*), YCB423 (*hst1*), YCB1097 (*hst2*), YCB470 (*hst3*), and YCB575 (*hst4*). These strains were all derived from FY2 strain background (Winston *et al.* 1995). (B) The strains containing *hst3 hst4* mutations grow poorly on SCFA-containing media. Strains spotted were (top to bottom): YCB173 (*sir2*), YCB515 (*hst1*), YCB405 (*hst3*), YCB523 (*hst4*), YCB235 (*sir2 hst1*), YCB538 (*hst3 hst4*), YCB547 (*hst3 hst4*), and YCB498 (*sir2 hst1 hst2 hst3 hst4*). These mutants were made in the YPH499/500 strain background (Sikorski and Hieter 1989).

when these strains were grown on plates with no additional carbon sources (Figure 6, YP0), with glycerol/ethanol (Figure 6, YPGE), or with glucose (data not shown). We tested the possibility that the closely related paralogous pairs, *SIR2/HST1* or *HST3/HST4*, had similar functions in SCFA metabolism. To examine this pos-

TABLE 3

Sirtuin-dependent activation of acetyl-CoA synthetase (Acs) enzyme function

Relevant genotype[a]	Omissions[b]	Additions	Acs specific activity[c]
acs$^+$ *cobB*$^+$	None		460 ± 0.057
	Mg(II)/ATP		95 ± 0.014
	Coenzyme A		<1
acs$^+$ *cobB*$^-$	None		<1
	None	10 μg CobB	42 ± 0.027
	None	10 μg CobB + NAD^+	490 ± 0.070
	Coenzyme A	10 μg CobB + NAD^+	<1

[a] All *S. enterica* strains also carried *metE205 ara-9 ack101::*MudJ mutations in the genome.
[b] The complete reaction mixture contained coenzyme A, Mg/ATP, and crude cell-free extract obtained from a strain with the indicated genotype.
[c] Specific activity is defined as picomoles of acetyl-CoA formed per minute per milligram of protein. Results are the average of three independent determinations.

sibility, we constructed and tested *sir2 hst1* and *hst3 hst4* double mutants (Figure 6B). The *sir2 hst1* mutant grew well on acetate and propionate. The *hst3 hst4* double mutant is more challenging to analyze due to its well-known genomic instability (Brachmann *et al.* 1995; Brachmann 1996). Many but not all isolates of *hst3 hst4* mutants showed a defect in growth on acetate and propionate. The extent of the defect varied from colony to colony. We tested five different *hst3 hst4* mutants from three different strain backgrounds (data not shown) and four of these showed growth defect on propionate. These experiments suggest that both *HST3* and *HST4* are involved in SCFA metabolism in yeast, as the corresponding single mutants showed no growth defects on SCFAs.

Sirtuin function is required to activate Acs in *S. enterica*: Table 3 shows evidence of sirtuin-dependent control of acetyl-CoA synthetase activity *in vitro*. The activity of acetyl-CoA synthetase in a sirtuin-deficient strain was undetectable. A 42-fold increase in activity was observed when homogeneous CobB sirtuin was added to the reaction mixture. The activity increased to 490-fold when excess NAD^+ was added to the sirtuin-containing reaction mixture. The level of acetyl-CoA synthetase activity obtained after treatment with CobB/NAD^+ was equivalent to the level of enzyme activity measured in cell-free extracts of the sirtuin-proficient strain. A control experiment with cell-free extract from a sirtuin-proficient strain carrying a deletion of the *acs* gene showed no detectable acetyl-CoA synthetase activity (data not shown). These results suggested that acetyl-CoA synthetase is activated by the CobB sirtuin in a NAD^+-dependent manner.

DISCUSSION

Data reported here support the conclusion that in *S. enterica* sirtuin function is required for the activation of acetate and propionate via the high-affinity acyl-CoA synthetase-dependent pathway of acyl-CoA synthesis. The data presented in Table 3 are consistent with the conclusion that the activity of acetyl-CoA synthetase and, by extension, propionyl-CoA synthetase, is controlled by their acetylation state. These data are consistent with the inability of sirtuin-deficient strains to use acetate or propionate as sources of carbon and energy. Evidence reported elsewhere shows that residue K609 of Acs is the site of acetylation. Residue K609 is an invariant residue in a motif that is conserved in many of the AMP-forming family of enzymes. In the case of Acs, K609 is essential for the formation of the acetyl-AMP intermediate, but is not required for the conversion of acetyl-AMP to acetyl-CoA (Starai *et al.* 2002). The effect that acetylation of K609 has on Acs activity is similar to the reported effect that a mutation in the residue equivalent to K609 of propionyl-CoA synthetase (*i.e.*, K592) has on the formation of propionyl-AMP and the conversion of the latter to propionyl-CoA (Horswill and Escalante-Semerena 2002). On the basis of these results, we hypothesize that acetylation is a common feature in the post-translational control of the biochemical activities of members of the AMP-forming family of enzymes.

A role for sirtuins in short-chain fatty acid metabolism beyond activation is unlikely since the lack of sirtuin function was completely bypassed by increasing the level of activity of the low-affinity acyl kinase/phosphotransacetylase pathway of acyl-CoA synthesis. These results are consistent with the explanation that the lack of sirtuin function blocks the synthesis of short-chain fatty acyl-CoA, not its utilization. Since inactivation of the *pta* gene did not affect growth of the *cobB*$^+$ *acs*$^+$ and *cobB*$^+$ *prpE*$^+$ strains on acetate or propionate, we conclude that Pta function is not part of the sirtuin-dependent pathway of short-chain fatty acid activation.

The involvement of sirtuins in short-chain fatty acid metabolism, in particular acetate metabolism, is of interest because of the prominent role of acetylated histones in eukaryotic chromatin silencing (Braunstein *et al.*

1993; Thompson *et al.* 1994). An effective way of controlling the degree of protein acetylation in the cell would be to control the level of acetyl-CoA substrate available to the acetyltransferase enzymes responsible for acetylation. It is reasonable to hypothesize that some substrate proteins for sirtuins may lose biological activity upon deacetylation; thus reactivation of these enzymes would depend on the level of acetyl-CoA available to the acetyltransferase enzymes. If this hypothesis is correct, then sirtuin-dependent stimulation of acetyl-CoA synthesis achieved by deacetylation of Acs becomes particularly important because active Acs enzyme would be essential to maintaining sufficient acetyl-CoA substrate concentration for the acetyltransferase enzymes to restore balanced levels of all the enzymatic activities under the control of this system.

The data reported here are consistent with the hypothesis that acetylated short-chain fatty acyl-CoA synthetases (PrpE and Acs) are inactive and that in the wild-type strain the deacetylase activity of sirtuins is responsible for keeping these enzymes active. It is also clear that in the absence of acetyl-CoA or propionyl-CoA synthetase activities, acetate and propionate are not retained inside the cell (Figure 5), suggesting that when the concentration of acetate or propionate in the environment is low, acyl-CoA synthetase activities are needed to retain these short-chain fatty acids inside the cell as their corresponding acyl-CoA derivatives.

Although there is no evidence in prokaryotes that sirtuins are involved in gene silencing or cell aging, sirtuins could still have a global effect on gene expression in prokaryotes. Downregulation of sirtuin activity under low-acetate growth conditions would result in low levels of acetyl-CoA with the concomitant reduction in the level of acetyl-phosphate, a known effector of gene expression (McCleary *et al.* 1993). The validity of the hypothesis presented above is under investigation.

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