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Biologically Active Isoforms of CobB Sirtuin Deacetylase in *Salmonella enterica* and *Erwinia amylovora*▽†

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Sirtuins are NAD^+-dependent protein deacylases that are conserved in all domains of life and are involved in diverse cellular processes, including control of gene expression and central metabolism. Eukaryotic sirtuins have N-terminal extensions that have been linked to protein multimerization and cellular localization. Here the first evidence of sirtuin isoforms in bacteria is reported. The enterobacterium *Salmonella enterica* synthesizes two isoforms of CobB sirtuin, a shorter 236-amino-acid isoform (here $CobB_S$) and a longer 273-amino-acid isoform (here $CobB_L$). The N-terminal 37-amino-acid extension of $CobB_L$ is amphipathic, containing 18 basic amino acids (12 of which are Arg) and 13 hydrophobic ones; both isoforms were active *in vivo* and *in vitro*. Northern blot and transcription start site analyses revealed that *cobB* is primarily expressed as two monocistronic *cobB* mRNAs from two transcription start sites, one of which was mapped within the neighboring *ycfX* gene and the other of which was located within *cobB*. Additionally, a low-abundance *ycfX-cobB* bicistronic mRNA was observed which could encode up to three proteins (YcfX, $CobB_L$, and $CobB_S$). $CobB_L$ isoforms are common within the family *Enterobacteriaceae*, but species of the genus *Erwinia* (including the plant pathogen *Erwinia amylovora*) encode only the $CobB_L$ isoform. The $CobB_L$ isoform from *E. amylovora* restored growth of as *S. enterica cobB* mutant strain on low acetate.

Homologues of the yeast silence information regulator 2 protein (ySir2p, also known as sirtuin) are Zn(II)-containing, NAD^+-dependent deacylases present in cells of all domains of life (21, 24, 35). In eukaryotes, sirtuin function impacts gene expression, metabolism, cancer, stress responses, and life span control (5, 18, 22, 29, 38). Ongoing work on the regulation of sirtuin activity levels has shown that sirtuins are regulated at the transcriptional, posttranscriptional, and posttranslational levels (22, 34).

In eukaryotes, some sirtuins contain N-terminal extensions of the enzymatic core, and these regions can affect the binding of interacting partners (11, 20), facilitate oligomerization of sirtuins (46), mediate interactions with other sirtuin forms (8, 27), and direct cellular localization (8, 31). Thus, these regions seem to contribute to the regulation of sirtuin function in the eukaryotic cell. It is not known whether sirtuin isoforms exist in bacteria or archaea or, if they do, what their functions may be.

Bacterial sirtuins play roles in short-chain fatty acid metabolism in *S. enterica* serovar Typhimurium LT2 (here *S. enterica*) and *Bacillus subtilis* (16, 17, 38) and in the catabolism of aromatic and allicyclic acids in *Rhodopseudomonas palustris* (10). In *S. enterica*, the protein acetyltransferase Pat acetylates acetyl coenzyme A (acetyl-CoA) synthetase (Acs) and propionylates propionyl-CoA synthetase (PrpE) (17, 37). In both cases, Pat acylates a conserved active-site lysine within the adenylation domain, effectively inactivating the enzyme by preventing the formation of the acetyl-AMP or propionyl-AMP intermediate (17, 36). Deacylation of propionylated PrpE ($PrpE^{Pr}$) or acetylated Acs (Acs^{Ac}) by the CobB sirtuin of *S. enterica* reactivates both enzymes, allowing cells to grow on propionate or low levels of acetate, respectively (17, 38).

The role of sirtuins in *S. enterica* central metabolism was recently expanded by data that suggest that a large number of central metabolic enzymes in *S. enterica* may be modified by reversible lysine acetylation (44). In addition to cellular metabolism, sirtuins have been linked to bacterial chemotaxis through the ability to deacetylate the response regulator CheY in *Escherichia coli* (26).

Though the role of bacterial sirtuins is expanding, little is known about the transcriptional regulation of the genes that encode these enzymes. Wang et al. reported the transcriptional profile of the *cobB* gene of *S. enterica* (44), but the signals that induce *cobB* expression and the location of the *cobB* promoter elements were not identified.

Based on the annotated *cobB* sequence of *S. enterica*, we sought to determine whether the CobB sirtuin contains an N-terminal extension to the sirtuin catalytic core, as predicted by bioinformatic analysis of the region. We also determined the transcription start site from which *cobB* mRNA is synthesized. Here, we provide evidence that *S. enterica* synthesizes two biologically active isoforms of CobB, one form of which contains a cationic 37-residue N-terminal extension. We used *in vitro* and *in vivo* approaches to demonstrate that each CobB isoform was active. We provide evidence that *cobB* is transcribed from multiple promoters and propose that these alternate transcripts may allow *S. enterica* to differentially regulate CobB isoform synthesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in nutrient broth (NB), no-carbon essential (NCE) minimal medium (3), or 3-(*N*-morpholino)propanesulfonic acid (MOPS) minimal medium (28). For Western blot assays, cells

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference[a]
S. enterica serovar Typhimurium LT2 strains		
JE6583	*metE205 ara-9*	Laboratory collection
JE7088	Δ*metE2702 ara-9*	Laboratory collection
Derivatives of JE6583		
JE10806	Δ*ycfX128*	
JE11228	Δ*cobB1375*	
Derivatives of JE7088		
JE12939	Δ*cobB1375* Δ*cobT1380*	Laboratory collection
JE12972	Δ*cobB1375* Δ*cobT1380*/pBAD30	
JE12973	Δ*cobB1375* Δ*cobT1380*/pCOBB8 (or $pCobB_{L/S}$)	
JE12974	Δ*cobB1375* Δ*cobT1380*/pCOBB19 (or $pCobB_L$)	
JE12975	Δ*cobB1375* Δ*cobT1380*/pCOBB24 (or $pCobB_S$)	
JE13302	Δ*cobB1375* Δ*cobT1380*/pCOBB93 (or p*Ea*$CobB_L$)	
E. coli JE6090	C41(λDE3)	Laboratory collection
Plasmids		
pBAD30	P_{araBAD} expression vector, *bla*$^+$	19
pTEV6	N-terminal, rTEV-cleavable MBP fusion overexpression vector, *bla*$^+$	30
pCOBB8 (or $pCobB_{L/S}$)	*cobB*$^+$ cloned into pBAD30, *bla*$^+$	38
pCOBB19 (or $pCobB_L$)	*cobB1372* allele ($CobB^{M37A\ M38A}$) in pBAD30, *bla*$^+$	
pCOBB24 (or $pCobB_S$)	*cobB1373* allele ($CobB^{M1A}$) in pBAD30, *bla*	
pCOBB93 (or p*Ea*$CobB_L$)	*E. amylovora cobB*$^+$ allele in pBAD30, *bla*$^+$	
pCOBB71	$CobB_S$ ORF in pTEV6	
pCOBB72	$CobB_L$ ORF in pTEV6	

[a] Unless otherwise indicated, all strains were constructed during the course of this work.

were grown using NCE minimal medium. For growth curves when acetate was the sole carbon source, MOPS minimal medium was supplemented with $MgSO_4$ (1 mM), L-methionine (0.5 mM), and acetate (10 mM, pH 7.0). For growth on glycerol, MOPS minimal medium was supplemented with $MgSO_4$ (1 mM), trace minerals (1×), dicyanocobinamide (15 nM), 5,6-dimethylbenzamidazole (125 μM), and glycerol (22 mM). When used, ampicillin was at 100 μg/ml. Cells were grown at 37°C with shaking. For growth curves, cell density was measured using a microtiter plate and a microtiter plate reader (Bio-Tek Instruments). Doubling times were calculated using the Prism 4 software package (GraphPad, La Jolla, CA).

Strain construction. Gene deletions in *S. enterica* were constructed by using the phage lambda Red recombinase system as described previously (12). Notably, the *ycfX* deletion was specifically designed to delete the 5′ portion (nucleotides [nt] 4 to 300) of the *ycfX* gene without disrupting putative *cobB* regulatory elements that exist in the 3′ region of the *ycfX* open reading frame (ORF).

Molecular techniques. DNA manipulations were performed using standard techniques (4, 14). Restriction endonucleases were purchased from Fermentas. DNA was amplified using PfuUltra II Fusion DNA polymerase (Stratagene), and site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmids were isolated using the Wizard Plus SV Miniprep kit (Promega), and PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). DNA sequencing was performed using BigDye (ABI PRISM) protocols, and sequencing reactions were resolved at the University of Wisconsin—Madison Biotechnology Center. The sequences of the oligonucleotide primers used are listed in Table 2.

Cloning of *S. enterica* and *Erwinia amylovora cobB* genes. Cloning of *S. enterica cobB* has been described previously (38). Site-directed mutagenesis of start codon M1 and M37/M38 yielded *cobB* alleles *cobB1372* (encodes $CobB^{M37A\ M38A}$ or $CobB_L$) on plasmid pCOBB19 (also referred to as $pCobB_L$) and *cobB1373* (encodes $CobB^{M1A}$ or $CobB_S$) on plasmid pCOBB24 (also referred to as $pCobB_S$).

The *E. amylovora cobB* gene was amplified from purified genomic DNA (a kind gift from N. Perna). The 1-kb fragment containing *E. amylovora cobB*$^+$ was digested with SacI and SphI and ligated into pBAD30 (19), which had been digested with the same enzymes. The resulting 6-kb plasmid was called pCOBB93 or p*Ea*$CobB_L$.

Protein purification. *S. enterica cobB* was amplified from JE6583 genomic DNA using primers to add a 5′ KpnI site and a 3′ SalI site to the amplification product. For $CobB_L$ overproduction, nt 1 to 822 of the *cobB* allele were amplified, and for $CobB_S$ overproduction, nt 109 to 822 of the *cobB* allele were amplified. Amplified fragments were digested with KpnI and SalI and subsequently ligated into plasmid pTEV6 (30) with the same enzymes. The resulting plasmids, pCOBB71 and pCOBB72, direct the synthesis of $CobB_S$ and $CobB_L$, respectively, with recombinant tobacco etch virus (rTEV) protease-cleavable N-terminal maltose-binding protein (MBP) tags.

Overproduction and purification methods were the same for both $CobB_L$ and $CobB_S$. Plasmids pCOBB71 and pCOBB72 were moved into *E. coli* strain C41(λDE3). The resulting strains were grown overnight and subcultured 1:100 (vol/vol) into 2 liters of super broth (13) containing ampicillin (100 μg/ml). Cultures were grown with shaking at 37°C to an A_{600} of ~0.7, and MBP-H_6-CobB synthesis was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM). The culture was grown overnight at 37°C. Cells were harvested at 8,000 × *g* for 15 min at 4°C in a Beckman Coulter Avanti J-2 XPI centrifuge fitted with a JLA-8.1000 rotor. Cell pellets were resuspended in 30 ml cold His-Bind buffer (buffer A, containing sodium phosphate buffer [20 mM, pH 7.5], NaCl [500 mM], and imidazole [20 mM]). Cells were placed on ice and lysed by sonication for 2 min (2-s pulse followed by 2 s of cooling) at level 7 in a model 550 sonic dismembrator (Fisher). The extract was cleared by centrifugation at 4°C for 30 min at 43,367 × *g*. Clarified cell extract was loaded onto a 5-ml HisTrap FF column (GE Healthcare) connected to a computer-controlled ÄKTA fast protein liquid chromatography (FPLC) system. Unbound proteins were eluted off the column by extensive washing with buffer A. A 65-ml wash step with 95% buffer A and 5% buffer B (sodium phosphate buffer [20 mM, pH 7.5], NaCl [500 mM], imidazole [500 mM]) was applied to the column prior to a 65-ml linear gradient of 5 to 100% buffer B. All fractions containing MBP-H_6-CobB were combined. rTEV protease (33) was added to MBP-H_6-CobB, and samples were dialyzed at room temperature for 3 h in buffer C (sodium phosphate buffer [20 mM, pH 7.5], NaCl [500 mM]) containing 1 mM dithiothreitol. CobB-rTEV mixtures were then dialyzed at 4°C against buffer C for 3 h and again against buffer C containing imidazole (20 mM) for 12 h. After cleavage and dialysis, protein mixtures were passed over the 5-ml HisTrap column following the protocol described above. Protein that did not bind to the column was analyzed by

TABLE 2. Primers and DNA probe used in this study

Name	Sequence[a]
cobB deletion primers	
cobB(L) Wanner 5′	GGTGCTGCTTTTTTACATCTTACCGACTAATCAAAAAAAGAGGTTGTTATGGTGTAGGCTGGAGCTGCTTC
cobB(L) Wanner 3′	ACGTAACGTGAAATGTAGGCCGGATAAGGCGTTACCGGGCAAACAGCACTACATATGAATATCCTCCTTAG
ycfX deletion primers	
ycfX1 Wanner 5′	GAGTATTGAGCGGCCAGTAATCGCGGAATATAAAAACAAGGACGGTGATGGTGTAGGCTGGAGCTGCTTC
ycfX1 Wanner 3′	ATCATCCCAGGCTTCGGACAAGGCAAAACAGTTAGCGTCATTGTCCAGACGCATATGAATATCCTCCTTAG
Cloning primers	
SacI + 5′cobB_Eamy	CAT**GAGCTC**CGCTTTGCTAAGGCGCG
SphI + 3′cobB_Eamy	ACTGCATGCTAAAGCGGGTTGGCCGTCG
KpnI + CobBL 5′ pTEV6	GTA**GGTACC**ATGCAGTCGCGTCGGTTTCATCG
KpnI + CobBS 5′ pTEV6	ATA**GGTACC**ATGGAAAACCCAAGAGTATTAGTCC
SalI + CobB 3′ pTEV6	GAT**GTCGAC**CAGCACTACAGCCCTTTCAGG
Site-directed mutagenesis primers	
SeCobB M1A F	CAAAAAAAGAGGTTGTTGCGCAGTCGCGTCG
SeCobB M1A R	CGACGCGACTGCGCAACAACCTCTTTTTTTG
SeCobB M37A, M38A F	GACAGAGTGGTGCCGGAAGCGGCGGAAAACCCAAGAG
SeCobB M37A, M38A R	CTCTTGGGTTTTCCGCCGCTTCCGGCACCACTCTGTC
5′ RACE analysis primers	
CobB_499_RT	CGCCGTTCCATTCCAGAATCTGG
CobB_435_RT	ATGAATGATGTTGCGATTGC
CobB_325_RT	GAT**AAGCTT**CCGCATTGGGTTGTATTTCC
Northern blot probes	
cobB_Northern_98-mer	CGGATAGACATGACCGGACGTGCCAATGGCGATAAAAATATCCGCCATCGACAGCGCCATATAAATTTCATCCATGCCAAGCGGCATCTCGCCAAACC
ycfX_Northern_96-mer	TCTAAAAAGGCGCTATAGCTGGTATGGGGCGTGGGAACCCGTTTTTCCGAGTGCAGCCGCCGCGTTGAGTCAAATACGCCTAACGCAATGTTTGTT

[a] Bold type indicates restriction sites.

sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) (23). Fractions containing $CobB_L$ or $CobB_S$ were pooled. $CobB_L$ and $CobB_S$ were stored in HEPES buffer (20 mM) containing NaCl (100 mM) and 10% glycerol. CobB concentrations were determined by measuring absorbance at 280 nm. The molar extinction coefficient used to calculate $CobB_L$ and $CobB_S$ concentrations was 22,460 M^{-1} cm^{-1}.

Western blot analysis. A 200-ml overnight culture grown in NB was washed twice with 1× NCE minimal medium. Cells were then inoculated into either NB or NCE minimal medium containing L-methionine (0.5 mM) and $MgSO_4$ (1 mM) and supplemented with acetate (50 mM), glycerol (50 mM), or glucose (50 mM) to an optical density at 650 nm (OD_{650}) of 0.1. For Western blot assays of citrate-grown cultures, cultures grown overnight in NB were inoculated to an OD_{650} of 0.025 into NCE minimal medium containing L-methionine (0.5 mM) and $MgSO_4$ (1 mM) and supplemented with citrate (50 mM) without washing. When cultures reached mid-log or stationary phase, they were placed on ice for 10 min to arrest growth and then centrifuged at 8,000 × *g* for 15 min at 4°C in a Beckman Coulter Avanti J-25I centrifuge fitted with a JLA-16.250 rotor. Cells were resuspended in HEPES buffer (50 mM, pH 7.5) containing phenylmethylsulfonyl fluoride (1 mM) and EDTA (10 mM). Cells were lysed by sonication on ice using a microtip at 50% duty for 1 min. Cell lysates were spun at 16,000 × *g* for 10 min at 4°C in an Eppendorf 5415D microcentrifuge to remove unbroken cells and cellular debris. Proteins in cellular extracts were quantified by absorbance at 280 nm. Fifty or 400 μg of cellular protein was resolved using SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore). Rabbit polyclonal CobB antiserum (Laboratory Animal Resources, University of Wisconsin—Madison) was used to detect isoforms of CobB. The CobB signal was visualized using alkaline phosphatase-conjugated goat anti-rabbit antibody (Pierce) and the one-step nitroblue tetrazolium—5-bromo-4-chloro-3′-indolylphosphate (NBT-BCIP) substrate according to the manufacturer's instructions.

CobB isoform abundance was quantified as described previously (43). Briefly, 2-fold dilutions of total cellular lysates (12.5 μg to 1.6 mg) prepared as described above were separated by SDS-PAGE using 1.5-mm-thick 12% polyacrylamide gels. Proteins were transferred and detected as described above. Band intensity was quantified using the TotalLab 1D gel analysis software v2003 (Nonlinear Dynamics, Durham, NC), and values within the linear range of detection were used to determine the relative CobB isoform levels. The values reported were in arbitrary units.

***In vitro* deacetylation assays.** Conditions for *in vitro* acetylation and deacetylation of Acs have been described previously (37). In the assays described here, we used homogeneous $CobB_L$, $CobB_S$, Pat, and Acs from *S. enterica* (17, 36, 37). Acs was radiolabeled using Pat protein acetyltransferase (37) and [1-^{14}C]acetyl-CoA in a final volume of 1 ml. Excess [1-^{14}C]acetyl-CoA was removed by buffer exchange; NAD^+ was added to the Acs^{Ac} reaction mixture to a final concentration of 1 mM, and deacetylation reactions were initiated by the addition of $CobB_L$ or $CobB_S$. Samples were periodically removed from the reaction mixture and quenched with SDS-PAGE loading buffer. Samples were resolved by SDS-PAGE and subjected to phosphorimaging analysis to assess the acetylation state of Acs after incubation with $CobB_S$ or $CobB_L$.

Oligomeric state analysis of $CobB_L$ and $CobB_S$. Gel filtration was performed using a HiPrep 26/60 Sephacryl S-100 high-resolution column (GE Healthcare) connected to a computer-controlled ÄKTA FPLC system. The column was equilibrated with sodium phosphate buffer (50 mM, pH 7.5) containing NaCl (150 mM). $CobB_L$ (0.8 mg) or $CobB_S$ (1 to 2 mg) was applied to the column, which was developed isocratically at a rate of 2 ml min^{-1}. Molecular weight calibrations were performed using the ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa) components of the Bio-Rad gel filtration standards. The above standards were supplemented with bovine serum albumin (66.4 kDa; Promega) and DNase I (31 kDa; Sigma).

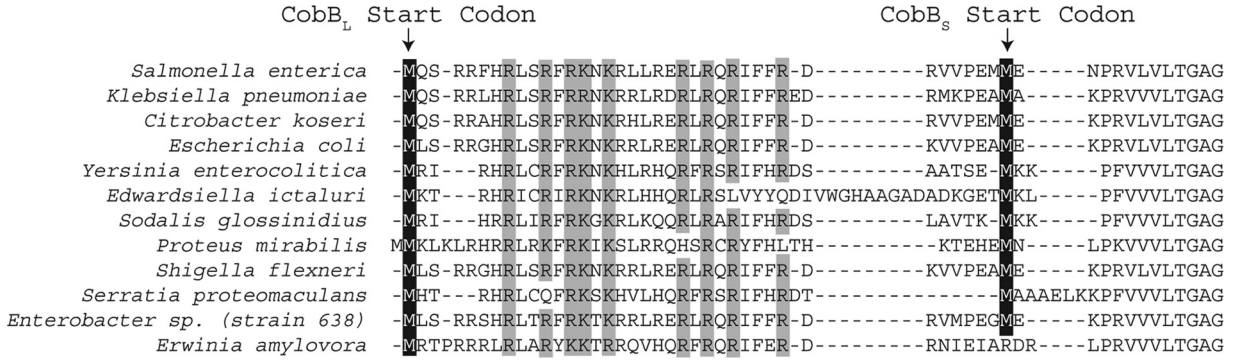

FIG. 1. CobB$_L$ and the positively charged N-terminal region are conserved among the members of the family *Enterobacteriaceae*. The N-terminal regions of CobB homologues from the family *Enterobacteriaceae* were aligned. The CobB$_L$ and CobB$_S$ starting methionines are highlighted in black. Conserved, positively charged residues (Arg and Lys) are highlighted in gray.

RNA isolation. For Northern blot analysis, RNA was isolated as described previously (1). *S. enterica* cultures were grown in NB to an OD_{650} of 0.8 with shaking at 37°C. A 520-μl volume of cells was added to 8× lysis buffer and phenol-water (3.75:1; Invitrogen), and the mixture was incubated at 65°C with shaking at 500 rpm for 20 min. Lysates were centrifuged at 16,000 × *g* for 10 min at room temperature in an Eppendorf 5415D microcentrifuge, and the aqueous phase was extracted twice with 700 μl phenol-chloroform-isoamyl alcohol (25:24:1; Ambion). RNA was precipitated with 1.3 ml ethanol (100%) at −80°C for at least 30 min. RNA was pelleted by centrifugation at 4°C for 10 min as described above. The pellet was washed with 500 μl ethanol (75%) and centrifuged again for 10 min at 4°C. RNA pellets were allowed to dry at room temperature, and RNA was resuspended in Tris-EDTA buffer, pH 8.0 (Ambion).

To perform 5′ rapid amplification of cDNA ends (5′ RACE) analysis, RNA was isolated using the RNA Protect reagent and the RNeasy Mini kit (Qiagen). Cells were grown to an OD_{650} of 0.7, and RNA was isolated according to the manufacturer's protocols. RNA was resuspended in RNase-free water.

Northern blot analysis. RNA (21 μg in 5.5 μl) was mixed with 1 μl MOPS buffer (10×), 3.5 μl formaldehyde, and 10 μl formamide (100%). RNA samples were incubated at 65°C for 15 min to denature the RNA. Samples were incubated on ice for 5 min, and 3 μl RNA agarose gel loading buffer (glycerol [50%], EDTA [1 mM], bromophenol blue [0.25%], xylene cyanol [0.25%]) was added. Samples were separated on a denaturing agarose gel (MOPS [1×], formaldehyde [2.2 M], agarose [1%]) with a Millennium marker (Ambion) run at 45 V for approximately 3 h. Gels were prepared for transfer as described previously (6).

RNA was transferred to 0.2-μm Hybond N^+ membrane (GE Healthcare) by capillary transfer for 16 h. RNA was linked to the membrane by baking for 2 h at 80°C. Membranes were stained for 45 s for total RNA with methylene blue (0.03%) in sodium acetate (0.3 M, pH 5.2). Membranes were then destained for 2 min with diethyl pyrocarbonate-treated water. Membranes were prehybridized with Ultrahyb (Ambion) at 42°C for at least 30 min. The membrane was probed overnight with a ^{32}P-radiolabeled single-stranded DNA probe (Table 2). Membranes were washed as described previously (6) and subjected to phosphorimager analysis to visualize the relative mobilities of the *cobB* and *ycfX* transcripts. The phosphorimager and methylene blue-stained membrane images were overlaid using Adobe Photoshop CS2 (Adobe Systems, Inc.) to determine the relative mobilities of the *cobB* and *ycfX* transcripts.

5′ RACE protocol. 5′ RACE was performed using the Version 2 5′ RACE system from Invitrogen according to the manufacturer's instructions. Nested, *cobB*-specific primers were used to synthesize the cDNA and for the subsequent amplification reactions. After the second round of amplification, the PCR products were separated by agarose gel electrophoresis and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). Fragments were sequenced using a *cobB*-specific primer to determine the 5′ end of the *cobB* transcript using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol. Reaction mixtures were resolved and analyzed at the University of Wisconsin Biotechnology Center.

RESULTS AND DISCUSSION

Multiple start codons of sirtuins are conserved in *Enterobacteriaceae*. An alignment of *cobB* homologues from representative members of the family *Enterobacteriaceae* suggested that most of these bacteria might synthesize two isoforms of CobB, a short (CobB$_S$) and a long (CobB$_L$) isoform (Fig. 1). The one exception we found was the plant pathogen *E. amylovora*, whose genome sequence appeared to direct the synthesis of only one form, CobB$_L$; this feature was conserved within all sequenced *Erwinia* species (data not shown). Notably, all of the N-terminal extensions of the putative CobB$_L$ isoforms synthesized by enterobacteria were amphipathic in nature, with many conserved basic residues (Arg and Lys, highlighted in gray), and in several instances, additional Arg residues were present in the extension but were not conserved in all genera. The extended peptide of the *S. enterica* CobB$_L$ isoform contained 17 basic amino acids (12 Arg, 2 Lys, 2 Gln, and 1 Asn; 17/37 = 46%) and 11 hydrophobic amino acids (4 Phe, 4 Leu, 2 Val, and 1 Ile; 11/37 = 30%).

***S. enterica* synthesizes short and long isoforms of CobB sirtuin.** The *cobB* gene of *S. enterica* contains two putative translation initiation sites within the first 38 codons (M1 and M38, respectively; Fig. 1). Although another potential start codon, M37, lies juxtaposed to M38, we considered M38 to be the biologically relevant start codon due to its conservation in other enterobacteria (Fig. 1). To determine whether different CobB isoforms are synthesized from the putative M1 and M38 start codons, we grew *S. enterica* in rich medium (NB) or in NCE minimal medium with acetate (50 mM) as the sole source of carbon and energy. Under all of these conditions, *S. enterica* expressed two isoforms of CobB which matched the predicted size of purified CobB standards (Fig. 2A). Samples were serially diluted, and densitometry was used to quantify the relative abundance of each isoform on Western blot assays. As shown in Fig. 2B, the shorter, 236-amino-acid CobB$_S$ isoform (start at M38) was expressed at levels approximately 7- or 11-fold higher than the longer 273-amino-acid CobB$_L$ isoform (start at M1) on NB and acetate, respectively. Previous work demonstrated that *cobB* is translated most highly at the mid-log and late-log stages of growth (44). Therefore, we assessed relative CobB$_L$ and CobB$_S$ levels during mid-log phase in *S. enterica* grown in NB or minimal medium supplemented with acetate, citrate, glucose, or glycerol (50 mM) (see Fig. S1 in the supplemental material). Under these conditions, CobB$_L$ levels were about 5- to 20-fold higher than CobB$_S$ levels (Fig. 1C).

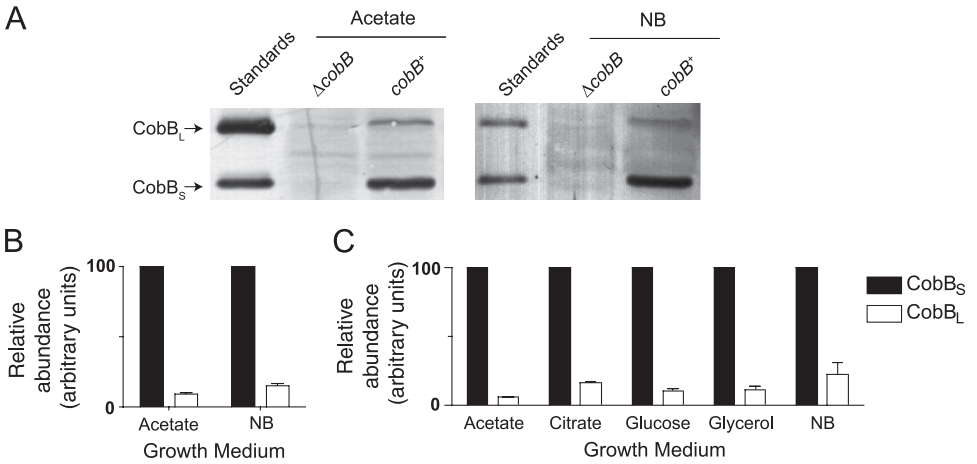

FIG. 2. *S. enterica* synthesizes two isoforms of CobB. Western blot analysis was performed using rabbit anti-CobB antiserum on whole-cell lysates of wild-type and Δ*cobB* mutant strains grown to stationary phase on NB and 50 mM acetate (A). The purified *Se*CobB$_L$ (31.1 kDa) and *Se*CobB$_S$ (26.2 kDa) proteins were included as molecular mass standards. Relative quantities of *Se*CobB$_L$ and *Se*CobB$_S$ were determined for cells grown to stationary phase (B) or mid-log phase (C) in NB or minimum medium supplemented with acetate, citrate, glucose, or glycerol (50 mM each). Quantities were normalized to *Se*CobB$_S$ levels, which were assigned a value of 100 arbitrary units.

The expression of these two CobB isoforms at differing levels suggested a previously unknown level of regulation of the CobB sirtuin in *S. enterica*.

The CobB$_L$ and CobB$_S$ isoforms deacetylate Acs^{Ac} *in vitro*. CobB sirtuin is the only annotated NAD$^+$-dependent deacetylase of *S. enterica*. Previous *in vitro* work with CobB$_S$ demonstrated that this isoform efficiently deacetylates acetylated acetyl-CoA synthetase (Acs^{Ac}) *in vitro* (36). However, prior to this work, the ability of CobB$_L$ to perform this reaction had not been assessed. To assess the deacetylase activity of CobB$_L$, *S. enterica* Acs was acetylated with radiolabeled acetyl-CoA, and the product of the reaction was used as the substrate for *in vitro* deacetylation activity assays. The decreasing amount of radiolabel associated with Acs over time showed that both CobB$_L$ and CobB$_S$ deacetylated Acs^{Ac} *in vitro* in a NAD$^+$-dependent manner (Fig. 3).

CobB$_L$ and CobB$_S$ deacetylate Acs^{Ac} *in vivo*. To determine whether CobB$_L$ and CobB$_S$ are active *in vivo*, we used sirtuin-deficient *S. enterica* strain JE12939 (Δ*cobB* Δ*cobT*). To assess the activity of the two CobB isoforms *in vivo*, we relied on the role of CobB during growth on 10 mM acetate (38). Under such conditions, acetate activation depends on Acs activity, which is regulated by reversible N$^\varepsilon$-Lys acetylation (36). The lack of *cobB* function in strain JE12939 prevents deacetylation and reactivation of Acs^{Ac} (36, 38). Therefore, growth of strain JE12939 on 10 mM acetate

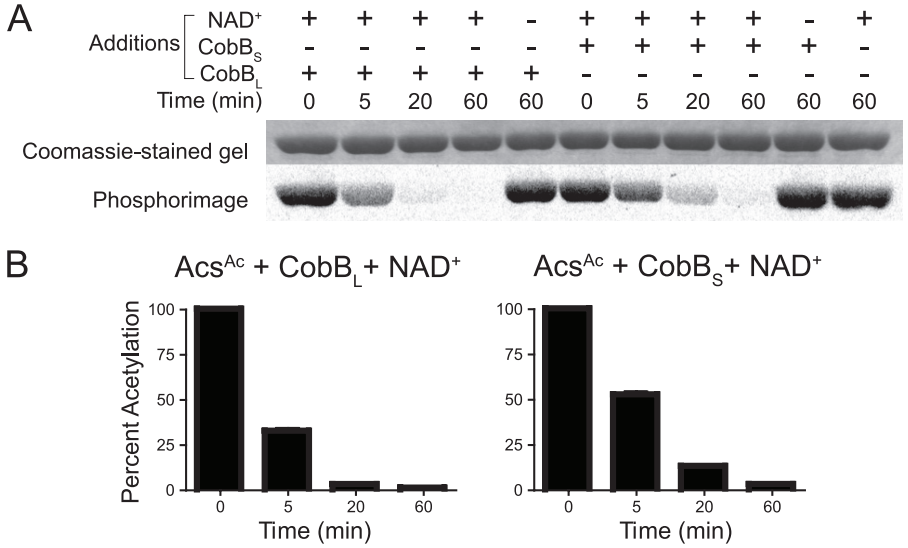

FIG. 3. *Se*CobB$_L$ and *Se*CobB$_S$ deacetylate Acs^{Ac} *in vitro*. *Se*CobB$_L$ or *Se*CobB$_S$ was incubated with NAD$^+$ and Acs^{Ac} that was previously acetylated by Pat and [1-^{14}C]acetyl-CoA. Samples were removed as a function of time and quenched with gel loading buffer. Proteins were resolved by SDS-PAGE and analyzed by phosphorimaging (A). The amount of radiolabeled Acs^{Ac} remaining at each time point was quantified by densitometry (B).

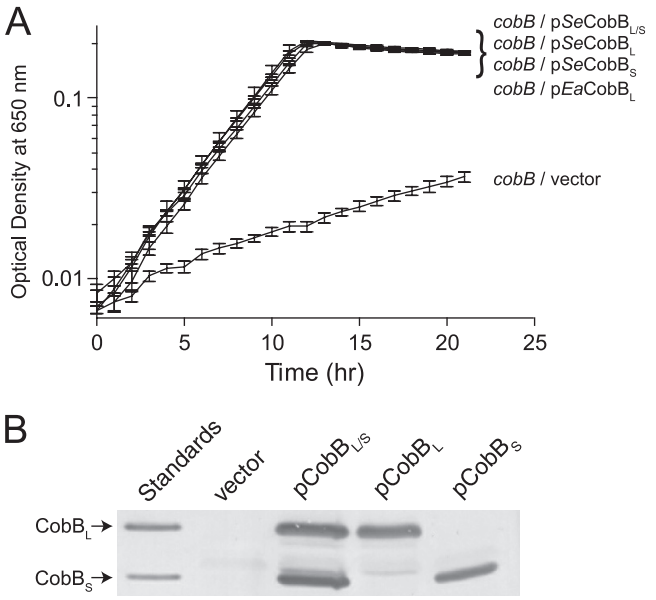

FIG. 4. *Se*CobB$_L$, *Se*CobB$_S$, and *Ea*CobB$_L$ proteins restore growth of an *S. enterica cobB* mutant strain on low acetate. (A) Growth behavior of *S. enterica* on MOPS minimal medium supplemented with acetate (10 mM). Growth experiments were performed at 37°C using a microtiter plate and a microtiter plate reader (Bio-Tek Instruments). (B) CobB Western blot analysis of *S. enterica cobB* strains overproducing *Se*CobB$_L$, *Se*CobB$_S$, or CobB$_{L/S}$ during growth on acetate (10 mM). *Se*CobB$_L$ is CobB$^{M37A\ M38A}$, encoded by *cobB1372*; *Se*CobB$_S$ is CobB^{M1A}, encoded by *cobB1373*.

would only occur if CobB$_L$ or CobB$_S$ were active, that is, if Acs^{Ac} were deacetylated.

To assess CobB$_S$ and CobB$_L$ activity *in vivo*, we introduced plasmids that encode arabinose-inducible alleles *cobB1372* (CobB$^{M37A\ M38A}$ = CobB$_L$) and *cobB1373* (CobB^{M1A} = CobB$_S$) into strain JE12939 (Δ*metE* Δ*cobB* Δ*cobT*). In Fig. 4A, we show that CobB$_L$ and CobB$_S$ restored growth of strain JE12939 on 10 mM acetate equally well. Western blot analysis confirmed that each plasmid expressed the expected CobB isoform (Fig. 4B). As expected, strain JE12939 failed to grow in the absence of CobB, a phenotype that was corrected by the introduction of the *cobB*$^+$ allele (positive control). Together, the data support the conclusion that CobB$_L$ and CobB$_S$ are functional *in vivo*.

To determine whether the ability of CobB$_L$ to deacetylate Acs^{Ac} was conserved among the members of the family *Enterobacteriaceae*, we asked whether the *E. amylovora cobB* gene could restore growth of *S. enterica* strain JE12939 on 10 mM acetate. As shown in Fig. 4, the *E. amylovora* CobB$_L$ (*Ea*CobB$_L$) protein supported growth of *S. enterica* strain JE12939 on 10 mM acetate.

The CobB$_L$ and CobB$_S$ isoforms have phosphoribosyltransferase activity. It has been known for some time that CobB compensates for the lack of the nicotinate mononucleotide: 5,6-dimethylbenzimidazole (DMB) phosphoribosyltransferase (CobT) enzyme during the synthesis of coenzyme B_{12} from its precursor cobinamide (40, 41). It was not known, however, whether both isoforms of CobB could compensate for CobT. Thus, we determined whether *S. enterica* CobB$_L$ (*Se*CobB$_L$), *Se*CobB$_S$, and *Ea*CobB$_L$ can restore adenosylcobalamin biosynthesis in a strain that carries a chromosomal deletion of the *cobT* gene. To ensure that the plasmid was the only source of CobB protein, we also deleted the chromosomal copy of *cobB*. Thus, the *S. enterica* JE12939 strain (Δ*metE* Δ*cobB* Δ*cobT*) used in this analysis was a cobalamin auxotroph because it cannot synthesize adenosylcobalamin from the precursors cobinamide and DMB. Hence, growth of strain JE12939 on glycerol-cobinamide-DMB would only occur if *Se*CobB$_L$, *Se*CobB$_S$, or *Ea*CobB$_L$ could activate DMB. Derivatives of strain JE12929 that synthesized *Se*CobB$_L$, *Se*CobB$_S$, or *Ea*CobB$_L$ grew on minimal medium supplemented with glycerol (22 mM), dicyanocobinamide (15 nM), and DMB (125 μM) at the following very similar doubling times: JE12939/p*Se*CobB$_L$, 1.2 ± 0.05 h; JE12939/p*Se*CobB$_S$, 1.1 ± 0.12 h; JE12939/p*Ea*CobB$_L$, 1.5 ± 0.04 h; JE12939/vector, 13.3 ± 0.04 h. The above data show that all of the forms of CobB tested activated DMB and restored adenosylcobalamin biosynthesis in an *S. enterica* strain lacking the CobT enzyme. Results from Western blot analysis confirmed that the plasmids used in this experiment expressed the expected CobB isoform (see Fig. S2 in the supplemental material).

Initial characterization of the CobB$_L$ N terminus. Previous work with the yeast sirtuin Hst2 demonstrated that an N-terminal extension to the core sirtuin domain could mediate sirtuin oligomerization (46). Gel filtration analysis of purified *Se*CobB$_L$ and *Se*CobB$_S$ demonstrated that both isoforms exist as monomers under the conditions tested (see Fig. S3 in the supplemental material). Thus, the N-terminal extension of *Se*CobB$_L$ does not mediate sirtuin oligomerization under the conditions tested.

Amphipathic helices have been demonstrated to allow the passage of small peptides through membranes (15). Because the N terminus of CobB is amphipathic in composition, we used secondary-structure prediction software to determine if this structure is capable of forming an amphipathic helix. The software package JPred3 (9) predicts that residues 6 to 28 form an alpha helix. However, a helical wheel projection of the predicted helix (see Fig. S4 in the supplemental material) suggested that this region of *Se*CobB$_L$ had charged residues evenly dispersed around the central axis of the helix and would not exist as a canonical amphipathic helix in which one face along the axis is hydrophobic while the other face is hydrophilic (32, 39).

In *S. enterica*, *cobB* is transcribed from two promoters independent of the neighboring *ycfX* gene. To gain insights into the mechanisms that control the synthesis of CobB isoforms in *S. enterica*, we investigated whether the cell synthesized ≥1 *cobB* mRNA. We note that, as currently ascribed, *cobB* is located 18 nt 3′ of *ycfX*, a homolog of the *nagK* gene that in *E. coli* encodes *N*-acetylglucosamine kinase (42). The proximity of *cobB* to *ycfX* suggested that *cobB* and *ycfX* might constitute an operon. In fact, information available online describes *nagK* (*ycfX*) *cobB* as an operon (http://regulondb.ccg.unam.mx/gene?term=ECK120003173&organism=ECK&format=jsp). Although Uehara and Park suggested that a promoter for *cobB* lies within *nagK*, the idea was not pursued (42).

We used Northern blot analysis to determine the length of the *cobB* transcript in *S. enterica*. If *cobB* (822 bp) and *ycfX* (912 bp) were cotranscribed, we expected a >1.7-kb transcript. Using a *cobB*-specific probe, we identified a dominant transcript approximately 1,000 nt in size, which suggested that the

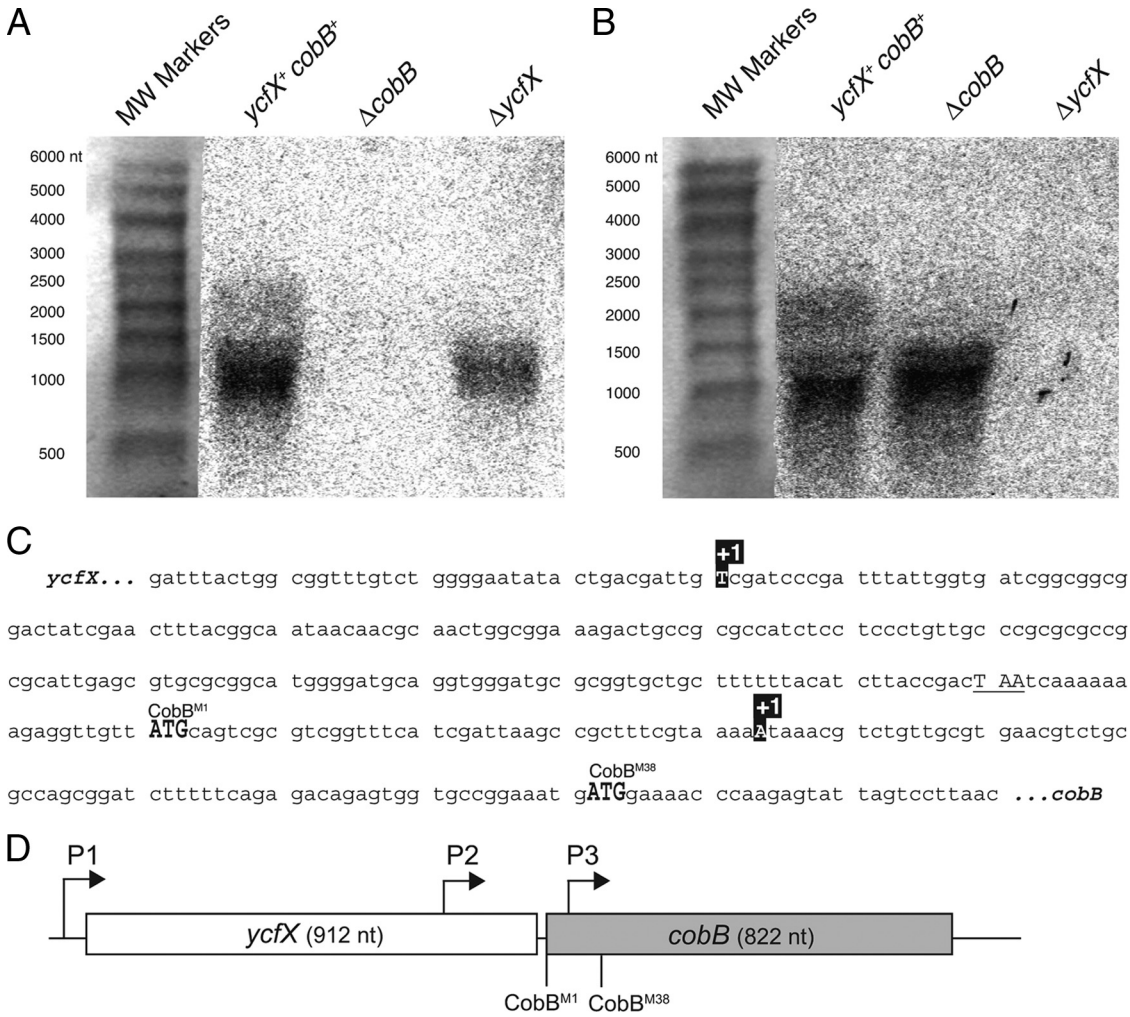

FIG. 5. The *cobB* gene has two promoters independent of the *ycfX* promoter. Northern blot assays of wild-type (*cobB*$^+$ *ycfX*$^+$) and Δ*cobB* and Δ*ycfX* mutant strains probing for *cobB* (A) or *ycfX* (B) transcripts. RNA Millennium markers were used as molecular weight standards. The phosphorimage was overlaid over the total RNA-stained membrane to align the lanes. (C) Identities of the *cobB* transcription start sites within the *ycfX* and *cobB* ORFs. The transcription start sites are designated +1. The CobB translation start sites are highlighted in gray. The *ycfX* stop codon is underlined. (D) Model of *cobB* promoters P1, P2, and P3 (drawn to scale). Assignment of promoters was based on Northern blot analysis (P1) or Northern blot analysis and transcription start site data (P2 and P3). The $CobB_L$ start codon is designated $CobB^{M1}$. The $CobB_S$ start codon is designated $CobB^{M38}$.

transcript spanned only *cobB* (Fig. 5A). Noteworthy was the small amount of signal appearing around 1,800 nt that was detected using *cobB*-specific and *ycfX*-specific probes (Fig. 5A and B) but was absent in either the Δ*ycfX* or the Δ*cobB* mutant strain, which suggested that a small amount of CobB was synthesized from a *ycfX-cobB* transcript.

We used 5′ RACE analysis to determine the transcription start sites of *cobB*. PCR amplification of the 5′ RACE products revealed two transcripts of different lengths. Although we refer to the 5′ mRNA ends identified from independent replicates as transcription start sites, the 5′ RACE protocol used does not distinguish between transcription start sites and mRNA processing sites. The sequences of these 5′ RACE products revealed a transcription site 5′ of each translation start site (Fig. 5C). One transcription start site was located within *ycfX* (nt 731), while the other was located within *cobB* (nt 44). The precise *cobB* transcription start site located within *ycfX* was somewhat ambiguous because the 5′ RACE protocol resulted in a poly(G) tail on the 5′ end of the transcript. It is possible that the G at the −1 site relative to the upstream *cobB* promoter could be the +1 nucleotide of the upstream *cobB* transcription start site.

A schematic of the *cobB* promoters is shown in Fig. 5D. Promoters were assigned based on the Northern blot analysis (for the *ycfX-cobB* promoter, P1) or Northern blot and 5′ RACE analyses (for the two promoters initiating monocistronic *cobB* mRNAs at P2 and P3). A small amount of *cobB* mRNA is transcribed from P1, and the P1-derived transcript contains the entire *cobB* ORF. The P2-derived transcript encompasses both of the ORFs that encode $CobB_L$ and $CobB_S$. $CobB_L$ is likely synthesized from the P2-derived transcript, but it is unclear whether or not $CobB_S$ is synthesized from this transcript. Promoter P3 exists within the *cobB* ORF. The P3-derived transcript only spans the ORF that encodes $CobB_S$,

suggesting that only $CobB_S$ can be synthesized from this transcript. By separating the expression of each *cobB* isoform with independent transcription start sites, *S. enterica* could differentially regulate each transcription start site and thus each CobB isoform.

Notably, when the *cobB* ORF is expressed from the P_{BAD} promoter, levels of $CobB_L$ and $CobB_S$ seem to be equalized (Fig. 4B) relative to when the *cobB* allele is expressed from the chromosome (Fig. 2A). The *cobB* complementation vector $pCobB_{WT}$ lacks the *cobB* P1 and P2 promoters but does contain the vector-based P_{BAD} promoter and *cobB* P3. Therefore, $CobB_L/CobB_S$ ratios seem to be regulated, in part, by the copy number of the *cobB* allele and/or the identity of the promoters driving *cobB* expression.

Contributions from this work. This is the first report of sirtuin isoforms in bacteria or archaea. The existence of sirtuin isoforms in a single bacterium suggests that these enzymes have more than one physiological role. We have shown that *S. enterica* synthesizes two biologically active isoforms of the CobB sirtuin deacetylase, which are synthesized from two sets of in-frame start codons. Long and short CobB sirtuin isoforms appear to be common among the members of the family *Enterobacteriaceae*, with the notable exception of the members of the genus *Erwinia*, which appear to synthesize only the long sirtuin isoform. Our data show that the $CobB_L$ isoform of *E. amylovora* is biologically active (Fig. 4). In *S. enterica*, the two CobB isoforms do not appear to be the result of processing of $CobB_L$ into $CobB_S$; instead, the isoforms are synthesized from alternative start codons. Examination of *cobB* gene expression revealed an additional level of regulation in which multiple *cobB* mRNAs are synthesized from transcription start sites located 5′ of each *cobB* translation initiation site (Fig. 5).

What roles could multiple isoforms of CobB be playing in *S. enterica*? Unique roles for $CobB_L$ and $CobB_S$ are not immediately obvious. It is striking that, among the bacterial $CobB_L$ isoforms we analyzed (Fig. 1), on average, 37% of the residues in the $CobB_L$ extension are Lys or Arg, with 78% of the positively charged residues being Arg. The structure of CobB from the enterobacterium *E. coli* has been determined (Protein Data Bank code 1S5P) (47); however, since the structure was solved using $CobB_S$, it is not clear what the structure of the N terminus of $CobB_L$ is or how it could be interacting with other macromolecules in the bacterial cell. Since *E. amylovora* is only capable of synthesizing $CobB_L$, it seem that this isoform is the biologically relevant isoform for this organism and is capable of carrying out all necessary $CobB_L$ functions in *E. amylovora*.

Although both isoforms of CobB can deacetylate Acs^{Ac}, Acs^{Ac} may not be the only physiologically relevant substrate for both isoforms. Recent work suggests that approximately 191 proteins in *S. enterica* are modified by acetylation (44), and putative protein partners of CobB in *E. coli* have been identified using a multiprotein complex purification procedure (7). The N-terminal region of CobB may be important for mediating interaction with some of these putative acetylated protein substrates. Alternatively, the positively charged N terminus of CobB may also be needed to interact with other molecules or structures in the cell. Nonribosomal, Arg-rich peptides with amino acid compositions similar to that of the N terminus of $CobB_L$ have been implicated in protein-RNA interactions (2, 25, 45). Thus, the N terminus of $CobB_L$ could bind nucleic acids or target the isoform to another negatively charged molecule of the cell. Thus, many questions remain unanswered. For example, (i) why are the levels of $CobB_L$ and $CobB_S$ different; (ii) does the positively charged N terminus of $CobB_L$ affect its activity, localization, or substrate specificity; and (iii) are there any physiological conditions under which either isoform is essential? These questions need to be addressed as we broaden our understanding of the physiological role of bacterial sirtuins.

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