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

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Sirtuins are NAD⁺-dependent protein deacetylases 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 CobB₆) and a longer 273-amino-acid isoform (here CobB₇). The N-terminal 37-amino-acid extension of CobB₇ 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, CobB₇, and CobB₆). CobB₇ isoforms are common within the family Enterobacteriaceae, but species of the genus Erwinia (including the plant pathogen Erwinia amylovora) encode only the CobB₆ isoform. The CobB₆ 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 (Sir2p, also known as sirtuin) are Zn(II)-containing, NAD⁺-dependent deacetylases 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 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 (PrpEPr) or acetylated AcS (AcSAc) 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...
were grown using NCE minimal medium. For growth curves when acetate was the sole carbon source, MOPS minimal medium was supplemented with MgSO₄ (1 mM), trace minerals (1×), dicyanocobinamide (15 mM), 5,6-dimethylbenzimidazole (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 recombinease 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[M1A M37A M38A] or CobB₃) on plasmid pCOBB19 (also referred to as pCobB₃) and cobB1373 (encodes CobB[M1A] or CobB₂) on plasmid pCOBB24 (also referred to as pCobB₂). 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 pEaCobB₂.

**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 CobB₃ overproduction, nt 1 to 822 of the cobB allele were amplified, and for CobB₂ 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 CobB₃ and CobB₂, 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 CobB₃ and CobB₂. Plasmids pCOBB71 and pCOBB72 were moved into E. coli strain C41(DE3). The resulting strains were grown overnight and subcultured 1:100 (vol/vol) into 2 liters of super broth (13) containing ampicillin (100 μg/ml). Cells were cultured at 37°C with shaking. Protein that did not bind to the column was analyzed by gel electrophoresis. Protein mixtures were then dialyzed at 4°C against buffer C for 3 h 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence**</th>
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<tbody>
<tr>
<td>cobB deletion primers</td>
<td></td>
</tr>
<tr>
<td>cobB(L) Wanner 5'</td>
<td>GGTTGCTGCTTTTTTACATCTTACGCCGACTAATCAAAAAAAAGGTGGTTATGCTTCTCCGAGTGGTACAGGGTTGGCCTGTCG</td>
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<tr>
<td>cobB(L) Wanner 3'</td>
<td>ACCTGAAGCTGAAATGTAOGCCGGGTAATAAGCCGGCGTTACCCGGCGGAAACACAGCATAATGGAATATCCTTCCTTG</td>
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<tr>
<td>ycfX deletion primers</td>
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<tr>
<td>ycfX Wanner 5'</td>
<td>GAGTTATTAGCCGCGCATTAATCGCGGAATAATATATACAAAGACGGTATGGAACGTTACGGTGTCATTGCCAGACGC</td>
</tr>
<tr>
<td>ycfX Wanner 3'</td>
<td>TATCGAATATCCCTCCTTAG</td>
</tr>
</tbody>
</table>

Cloning primers

Sacl + 5’cobB_Eamy         | CATTAGCGCTCCGTTCGTTGCTAGGCGC |
SphI + 3’cobB_Eamy         | ACTGCTAGCTCAAAAGGGTGTGGCCTG |
KpnI + CobBL 5’pTEV0       | GTAGTTGACCATGAGTGCGTGGTTCATG |
KpnI + CobBS 5’pTEV0       | ATAGTGACTGTAAAACCCAGAAGTAGTATTC |
SalI + CobB 3’pTEV0        | GATGGCAGCACACTACAGCCCTTCCAG |

Site-directed mutagenesis primers

SeCobB M1A F              | CAAAACAAAGGAGGTGTTGCTAG |
SeCobB M1A R              | CGACGCGGACTGCGCAACAACCTCTTTTTTG |
SeCobB M37A, M38A F       | GACAGAGTGGTCGGAGAGCAG |
SeCobB M37A, M38A R       | CTCTTGGTTTCCCCGGTCGCCACCTTCGGTC |

5′ RACE analysis primers

CobB_499 RT              | CGCCGTTCTCATCCGACCATG |
CobB_435 RT              | ATGAAGTATGTTGCGATTGC |
CobB_325 RT              | GATAGCTTGCTTCGCAATGGGTGTTATTC |

Northern blot probes

cobB_Northern_98-mer      | CGGATAGAATGACAGCCGCGACGCGCTTACGGGATAAAATATCCGCCGCAATCACAGCACTACAGCCCTTTCG |
ycfX_Northern_96-mer      | TCTAAAAAACCCGCTATAGCTGTTATGGGGCGTTGGGAACCCGGGTTTCCGAGT |
GCAGCCCGCGCCGTTTAGCTTAAAATACGCTAACAGGAATGTTG |

**Bold type indicates restriction sites.
RNA isolation. For Northern blot analysis, RNA was isolated as described previously (1). S. enterica cultures were grown in NB to an OD_605 of 0.8 with shaking at 37°C. A 520-μl volume of cells was added to 8x lysis buffer and phenol-water (3:7:5; 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). Reaction mixtures were re-sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer’s protocol. Reaction mixtures were resolved and analyzed by 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_S; this feature was conserved within all sequenced Erwinia species (data not shown). Notably, all of the N-terminal extensions of the putative CobB_S 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 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).

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_S and CobB_L 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_S levels were about 5- to 20-fold higher than CobB_L levels (Fig. 1C).

FIG. 1. CobB_S 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 are aligned. The CobB_L and CobB_S start methionines are highlighted in black. Conserved, positively charged residues (Arg and Lys) are highlighted in gray.
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<sub>L</sub> and CobB<sub>S</sub> isoforms deacetylate Acsc<sup>Ac</sup> in vitro.** CobB sirtuin is the only annotated NAD<sup>+</sup>-dependent deacetylase of *S. enterica*. Previous in vitro work with CobB<sub>S</sub> demonstrated that this isoform efficiently deacetylates acetylated acetyl-CoA synthetase (Acsc<sup>Ac</sup>) in vitro (36). However, prior to this work, the ability of CobB<sub>L</sub> to perform this reaction had not been assessed. To assess the deacetylase activity of CobB<sub>L</sub>, *S. enterica* Acsc 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 Acsc over time showed that both CobB<sub>L</sub> and CobB<sub>S</sub> deacetylated Acsc<sup>Ac</sup> in vitro in a NAD<sup>+</sup>-dependent manner (Fig. 3).

**CobB<sub>L</sub> and CobB<sub>S</sub> deacetylate Acsc<sup>Ac</sup> in vivo.** To determine whether CobB<sub>L</sub> and CobB<sub>S</sub> are active in vivo, we used sirtuin-deficient *S. enterica* strain JE12939 (ΔcobB ΔcobT). 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 Acsc activity, which is regulated by reversible Nε-Lys acetylation (36). The lack of cobB function in strain JE12939 prevents deacetylation and reactivation of Acsc<sup>Ac</sup> (36, 38). Therefore, growth of strain JE12939 on 10 mM acetate...
would only occur if CobB\(_L\) or CobB\(_S\) were active, that is, if Acs\(^{Ac}\) were deacetylated.

To assess CobB\(_L\) and CobB\(_S\) activity in vivo, we introduced plasmids that encode arabinosin-inducible alleles cobB1372 (CobB\(^{M37A\, M38A}\) = CobB\(_L\)) and cobB1373 (CobB\(^{M1A}\) = CobB\(_S\)) into strain JE12939 (\(\Delta\)metE \(\Delta\)cobB \(\Delta\)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\(_L\) (EaCobB\(_L\)) 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\(_L\) (ScCobB\(_L\)), ScCobB\(_S\), and EaCobB\(_L\) can restore adenosylcobalamin bio-synthesis 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 (\(\Delta\)metE \(\Delta\)cobB \(\Delta\)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 glyc erol-cobinamide-DMB would only occur if ScCobB\(_L\), ScCobB\(_S\), or EaCobB\(_L\) could activate DMB. Derivatives of strain JE12929 that synthesized ScCobB\(_L\), ScCobB\(_S\), or EaCobB\(_L\) grew on minimal medium supplemented with glyc erol (22 mM), dicyanocobinamide (15 nM), and DMB (125 \(\mu\)M) at the following very similar doubling times: JE12939/pSeCobB\(_L\), 1.2 \(\pm\) 0.05 h; JE12939/pSeCobB\(_S\), 1.1 \(\pm\) 0.12 h; JE12939/pEaCobB\(_L\), 1.5 \(\pm\) 0.04 h; JE12939/vector, 13.3 \(\pm\) 0.04 h. The above data show that all of the forms of CobB tested activated DMB and restored adenosylcobalamin biosyn thesis 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 ScCobB\(_L\) and ScCobB\(_S\) demonstrated that both isoforms exist as monomers under the conditions tested (see Fig. S3 in the supplemental material). Thus, the N-terminal extension of ScCobB\(_L\) 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 CobB 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 \(\approx 1\) cobB mRNA. We noted that, as currently ascribed, cobB is located 18 nt 3' of ycfX, a homolog of the nagK gene that in E. coli encodes N-acetylglu coseamine 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=jpg). 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 \(\approx 1.7\)-kb transcript. Using a cobB-specific probe, we identified a dominant transcript approximately 1,000 nt in size, which suggested that the

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**FIG. 4.** ScCobB\(_L\), ScCobB\(_S\), and EaCobB\(_L\) 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 ScCobB\(_L\), ScCobB\(_S\), or CobB\(_L\)\(_S\) during growth on acetate (10 mM). ScCobB\(_S\) is CobB\(^{M37A\, M38A}\), encoded by cobB1372; ScCobB\(_L\) is CobB\(^{M1A}\), encoded by cobB1373.
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 ycfX or the 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 analysis and transcription start site data (P2 and P3). The CobB_M1 start codon is designated CobB^{M1}. The CobB_M38 start codon is designated CobB^{M38}.
putative protein partners of CobB in CobBL have been implicated in protein-RNA interactions (2, amino acid compositions similar to that of the N terminus of substrates. Alternatively, the positively charged N terminus of ing interaction with some of these putative acetylated protein 

fied using a multiprotein complex purification procedure (7). The cobB complementation vector pCobBWT lacks the 

is the biologically relevant isoform for this organism and is independent transcription start sites, 

pCobBWT lacks the 

VOL. 192, 2010 BACTERIAL SIRTUIN ISOFORMS 6207 

in-frame start codons. Long and short CobB sirtuin isoforms 

have more than one physiological role. We have shown that 

are modified by acetylation (44), and 

ally based PBAD promoter and cobB P3. Therefore, 

cobB allele is expressed from the chromosome (Fig. 2A). The cobB complementation vector lacks the cobB P1 and P2 promoters but does contain the vector-based PBAD promoter and cobB P3. Therefore, 

obtain the vector-based PBAD promoter and 

amylovora is biologically active (Fig. 4). In S. enterica, the two CobB isoforms do not appear to be the result of processing of CobB into CobB, 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 and CobB are not immediately obvious. It is striking that, among the bacterial CobB isoforms we analyzed (Fig. 1), on average, 37% of the residues in the CobB 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 (Pro- 

tein Data Bank code 1SSP) (47); however, since the structure was solved using CobB, it is not clear what the structure of the N terminus of CobB is or how it could be interacting with other macromolecules in the bacterial cell. Since E. amylovora is only capable of synthesizing CobB, it seems that this isoform is the biologically relevant isoform for this organism and is capable of carrying out all necessary CobB functions in E. amylovora. 

Although both isoforms of CobB can deacetylitate AcAcs, AcAcs 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 have been implicated in protein-RNA interactions (2, 72). Thus, the N terminus of CobB 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 and CobB different; (ii) does the positively charged N terminus of CobB 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 bacte- 

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