PanM, an Acetyl-Coenzyme A Sensor Required for Maturation of L-Aspartate Decarboxylase (PanD)

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ABSTRACT **Coenzyme A (CoA) is essential for cellular chemistry in all forms of life. The pantothenate moiety of CoA is generated from the condensation of pantoate and β-alanine. β-Alanine is formed by decarboxylation of L-aspartate catalyzed by PanD, a pyruvoyl enzyme that is synthesized by the cell as an inactive precursor (pro-PanD). Maturation of pro-PanD into PanD occurs via a self-cleavage event at residue Ser25, which forms the catalytic pyruvoyl moiety. We recently reported that *Salmonella enterica* PanM was necessary for pro-PanD maturation, both *in vitro* and *in vivo*. Notably, PanM is annotated as a Gcn5-like *N*-acetyltransferase (GNAT), which suggested that lysine acetylation might be part of the mechanism of maturation. Here we show that PanM lacks acetyltransferase activity and that acetyl-CoA stimulates its activity. Results of experiments with nonhydrolyzable ethyl-CoA and genetically encoded acetyl-lysine-containing PanD support the conclusion that PanM-dependent pro-PanD maturation does not involve an acetyl transfer event. We also show that CoA binding to PanM is needed for *in vivo* activity and that disruption of CoA binding prevents PanM from interacting with PanD. We conclude that PanM is a GNAT homologue that lost its acetyltransferase activity and evolved a new function as an acetyl-CoA sensor that can trigger the maturation of pro-PanD.**

IMPORTANCE **Nε-lysine acetylation is increasingly being recognized as a widespread and important form of posttranslational regulation in bacteria. The acetyltransferases that catalyze these reactions are poorly characterized in bacteria. Based on annotation, most bacterial genomes contain several acetyltransferases, but the physiological roles of only a handful have been determined. Notably, a subset of putative acetyltransferases lack residues that are critical for activity in most biochemically characterized acetyltransferases. We show that one such putative acetyltransferase, PanM (formerly YhhK), lacks acetyltransferase activity but functions instead as an acetyl-coenzyme A (CoA) sensor. This work establishes the possibility that, like PanM, other putative acetyltransferases may have evolved new functions while retaining the ability to sense acetyl-CoA.**

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Coenzyme A (CoA) is essential for all forms of life, with an estimated 4% of known enzymes requiring it for function (1). CoA is generated from the precursor pantothenate (1). We recently reported evidence that PanM (formerly YhhK) triggers the maturation of the L-aspartate decarboxylase (PanD; EC 4.1.1.11) enzyme of *Salmonella enterica* (2). The product of the PanD-catalyzed reaction is β-alanine (3), which is one of four steps comprising the pantothenate biosynthesis pathway (4). PanD is a pyruvoyl enzyme that is translated as an inactive proprotein (pro-PanD; 14 kDa), which must be cleaved into a 3-kDa β subunit and an 11-kDa α subunit whose N terminus is a pyruvoyl moiety required for activity (5). Pro-PanD catalyzes its own cleavage under nonphysiological conditions, such as at 50°C (5). However, PanM is necessary for efficient cleavage at 37°C, both *in vivo* and *in vitro* (2). The mechanism by which PanM accelerates PanD maturation remains unknown.

Based strictly on its homology to yGcn5p (EC 2.3.1.48), the PanM protein of *Salmonella enterica* and *Escherichia coli* was annotated as an acetyltransferase belonging to the Gcn5-like *N*-acetyltransferase (GNAT) family. Acetyltransferases catalyze the transfer of an acetyl moiety from acetyl-CoA to their protein or small molecule substrates, generating *O*- or *N*-acylated products (6). The idea of PanM regulating PanD by acetylation was attractive because acetyltransferases use acetyl-CoA as a substrate and PanD catalyzes one of the first steps of the pantothenate synthesis pathway whose ultimate product is CoA (4, 7). The cellular pool of CoA is a mixture of acylated and nonacylated (CoASH) coenzyme, with acetyl-CoA being the dominant acyl-CoA species under most growth conditions (8, 9). If PanM were an acetyltransferase, high levels of acetyl-CoA could drive PanM activity, resulting in more PanD being activated and ultimately increased synthesis of free CoA.

Although PanM is annotated as a putative GNAT, no evidence of acetyltransferase activity associated with PanM has been reported (2). In most characterized protein acetyltransferases, the catalytic base is a conserved glutamate residue, which deprotonates the epsilon amino group of the target lysine on the substrate protein, triggering a nucleophilic attack on the carbonyl group of

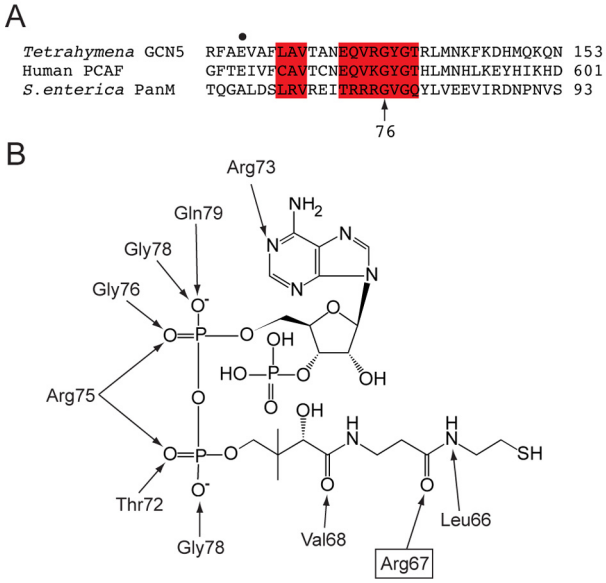

FIG 1 CoA-binding site of PanM. (A) Alignment of CoA-binding motif (motif A; highlighted in red) from *Tetrahymena* GCN5, human PCAF, and *S. enterica* PanM. The catalytic glutamate of tGCN5 and hPCAF is indicated by a black dot. PanM residue Gly76 is identified by an arrow. (B) Residues of PanM that interact with CoA. Side chain interactions are boxed; all other interactions involve main chain atoms.

acetyl-CoA. The result of this attack is the transfer of the acetyl moiety of acetyl-CoA onto the target lysine residue (6, 10). Notably, PanM lacks the catalytic glutamate (Fig. 1A), suggesting that PanM does not have acetyltransferase activity or that an alternative residue is used as a base. Although PanM may not have acetyltransferase activity, it still binds CoA, as shown by the structure of *Escherichia coli* PanM in complex with CoA (J. R. Cort, A. Yee, C. H. Arrowsmith, and M. A. Kennedy, unpublished data; PDB 2K5T). Here we have shown that acetyl-CoA stimulates *S. enterica* PanM activity, accelerating PanD cleavage by an acetylation-independent mechanism. We propose that PanM acts as an acetyl-CoA sensor, and we discuss the implications of this idea in the context of CoA homeostasis.

RESULTS

PanM activity is stimulated by acetyl-CoA. The ability of PanM to bind CoA has been demonstrated by a solution nuclear magnetic resonance (NMR) structure of *E. coli* PanM bound to CoA (J. R. Cort, A. Yee, C. H. Arrowsmith, and M. A. Kennedy, unpublished data; PDB 2K5T). To determine whether the CoA-binding motif of PanM resembled that of other acetyltransferases, PanM was aligned with two *bona fide* acetyltransferases of the GNAT family (e.g., *Tetrahymena* GCN5 and human PCAF) (Fig. 1A). Residues predicted to bind CoA in the alignment were verified using the PanM-CoA NMR structure (Fig. 1B). Although only 5 of 11 residues comprising the CoA binding motifs of the GNATs were conserved in PanM (Fig. 1A), the core RGXG motif was present (11). Similar to other GNATs (11), all but one of the interactions with CoA (Arg67) involved main-chain PanM atoms (Fig. 1B).

Since acetyl-CoA is the cosubstrate of other GNATs, the ability of PanM to bind CoA prompted the question of whether acetyl-CoA had any effect on PanM activity. Pro-PanD was preincubated with PanM or with PanM and acetyl-CoA, allowing PanM to activate pro-PanD maturation. PanD activity was then determined by high-performance liquid chromatography (HPLC) analysis. After preincubation with PanM and acetyl-CoA, a modest but reproducible increase in PanD activity was noted (~2.1-fold; $P < 0.0001$) relative to the activity measured when pro-PanD was incubated with PanM alone (Fig. 2A, bars 1 and 3). Under the experimental conditions used, acetyl-CoA stimulated PanM activity. As a control, pro-PanD was preincubated with PanM and CoASH (Fig. 2A, bar 2). Although a slight stimulation of PanD activity was observed compared to that for PanD incubated with PanM alone (~1.3-fold), the above results suggested that acetyl-CoA might be the physiological signal that stimulates PanM activity *in vivo*.

PanM does not transfer an acetyl moiety to PanD. The effect of acetyl-CoA on PanM-dependent pro-PanD maturation left open the possibility that PanM was an acetyltransferase. To facilitate acetylation studies, ethyl-CoA, a nonhydrolyzable analog of acetyl-CoA, was synthesized and purified to >99% homogeneity (see Fig. S1 in the supplemental material). If transfer of the acetyl moiety from acetyl-CoA were part of the mechanism of action of

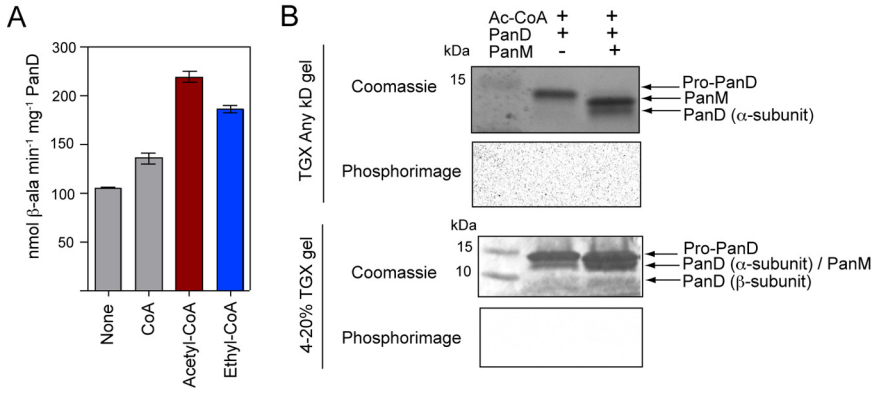

FIG 2 Acetyl-CoA stimulates PanM activity by an acetyltransferase-independent mechanism. (A) Specific activities of PanD after preincubation with PanM alone (bar 1; gray), PanM with CoA (bar 2; gray), PanM with acetyl-CoA (bar 3; red), or PanM with ethyl-CoA (bar 4; blue). (B) Acetylation assays containing [1-^{14}C]acetyl-CoA and pro-PanD alone (reaction 1) or pro-PanD with PanM (reaction 2). Proteins were visualized using Coomassie brilliant blue dye, and acetylation was detected by phosphorimaging. Proteins present in acetylation reaction mixtures were resolved using a Bio-Rad TGX Any kD gel (top panels) to visualize the C-terminal α fragment of mature PanD or a Bio-Rad 4 to 20% TGX gel to visualize the N-terminal β-fragment (bottom panels).

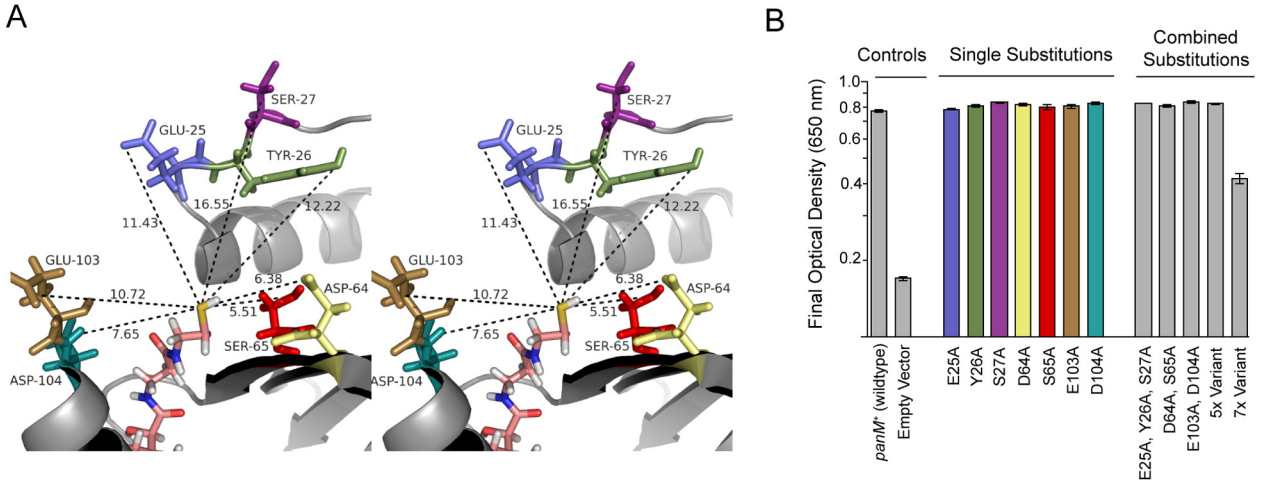

FIG 3 Alanine scan of PanM. Potential catalytic residues are E25 (blue), Y26 (green), S27 (purple), D64 (yellow), S65 (red), E103 (brown), and D104 (teal). (A) Stereo view of PanM bound to CoA (PDB 2K5T). Distances (Å) between each residue and the sulfur atom of CoA are indicated. CoA is colored by atom: C (pink), S (yellow), H (white), N (blue), and O (red). (B) Complementation of a Δ*panM S. enterica* strain (JE12555) with *panM* genes encoding alanine substitutions at each potential catalytic residue. Final optical densities are shown for strains grown for 15 h on minimal glycerol medium lacking β-alanine. 5× variant, $PanM^{E25A, Y26A, S27A, D64A, S65A}$; 7× variant, $PanM^{E25A, Y26A, S27A, D64A, S65A, E103A, D104A}$.

PanM, we reasoned that substitution of ethyl-CoA for acetyl-CoA in the reaction mixture should substantially reduce PanM-dependent pro-PanD maturation. As shown in Fig. 2A, PanM activity was stimulated by ethyl-CoA (bar 1 versus bar 4). Although the level of stimulation was less than the level observed in the presence of acetyl-CoA (bar 3), the difference in activity between pro-PanD incubated with PanM and ethyl-CoA was significantly higher ($P < 0.0001$) than that of pro-PanD incubated with PanM alone. The fact that ethyl-CoA stimulated PanM activity indicated that hydrolysis of the acyl group was not necessary for acetyl-CoA stimulation of PanM activity.

We looked further into the possibility of PanM having acetyltransferase activity using a radiometric acetyltransferase assay to investigate whether an acetyl transfer to PanD occurred during PanM-dependent pro-PanD maturation. In such experiments, [1-^{14}C]acetyl-CoA was incubated either with pro-PanD alone or with pro-PanD and PanM, and two different SDS-PAGE systems were used to resolve proteins in the reaction mixtures. The Any kD TGX gel (Bio-Rad) was used to distinguish the C-terminal α subunit of mature PanD from pro-PanD (2). A 4 to 20% TGX gel (Bio-Rad) was used to visualize the small N-terminal β-subunit of PanD. The β-subunit was difficult to visualize in the gel due to inefficient staining, so the band corresponding to the PanD β-subunit was excised from the gel, and its identity was established using matrix-assisted laser desorption–ionization time-of-flight/time-of-flight (MALDI TOF-TOF) mass spectrometry (see Fig. S2 in the supplemental material). In reactions where PanM-dependent maturation of pro-PanD was observed, no transfer of radiolabeled acetyl moieties onto either subunit of mature PanD was detected (Fig. 2B). In addition, nearly complete cleavage of PanD was observed in the presence of acetyl-CoA (Fig. 2B) compared to the ~50% cleavage previously reported for PanD incubated with PanM in the absence of acetyl-CoA (2). These data confirm that acetyl-CoA stimulates PanM-dependent maturation of pro-PanD. Collectively, the lack of acetyl transfer onto PanD and the ability of ethyl-CoA to stimulate PanM activity strongly supported an acetylation-independent mechanism for PanM function.

Does PanM have an alternative catalytic base for acetyl transfer? In Gcn5 and PCAF, a catalytic glutamate residue is located 10.6 Å and 11.5 Å, respectively, from the sulfur atom of CoA (12, 13). PanM has an alanine residue in this position (Fig. 1A, circle), suggesting that PanM may lack acetyltransferase activity. To determine whether any other residues may be acting as the catalytic base, we changed all charged residues within 12 Å of the sulfur atom of CoA to alanines (Fig. 3A). The effects of these changes on PanM function were determined individually or in combination by assessing the functionality of each variant *in vivo*. For this purpose, each plasmid was transformed into an *S. enterica* Δ*panM* strain, and growth in minimal glycerol medium in the absence of β-alanine was measured after 15 h at 37°C. Surprisingly, reduced growth was observed only for the plasmid encoding a variant PanM containing all seven substitutions (Fig. 3B). These data suggested that none of the seven charged residues in the proximity of the CoA sulfur atom functioned as a catalytic base for acetyl transfer.

Acetylated pro-PanD cannot self-cleave in the absence of PanM. To further investigate whether or not PanM activated pro-PanD by acetylation, we ascertained the effect of acetylation on the activity of PanD. To do this, we engineered a set of plasmids to produce six pro-PanD variants, each of which had acetyl-lysine in lieu of lysine at position 9, 14, 53, 104, 115, or 119. Each plasmid was expressed in both an *S. enterica* Δ*panD* strain to assess *in vivo* PanD activity and an *S. enterica* Δ*panM* strain to determine whether pro-PanD acetylated at the above-mentioned positions could self-process in the absence of PanM. As expected, the control plasmid producing nonacetylated pro-PanD complemented the Δ*panD panM*$^+$ strain on minimal medium devoid of β-alanine but did not restore pantothenate biosynthesis in the Δ*panM panD*$^+$ strain (Fig. 4A). In contrast, all plasmids producing acetylated pro-PanD variants failed to restore growth of the Δ*panD panM*$^+$ and Δ*panM panD*$^+$ strains (Fig. 4A). All acetylated

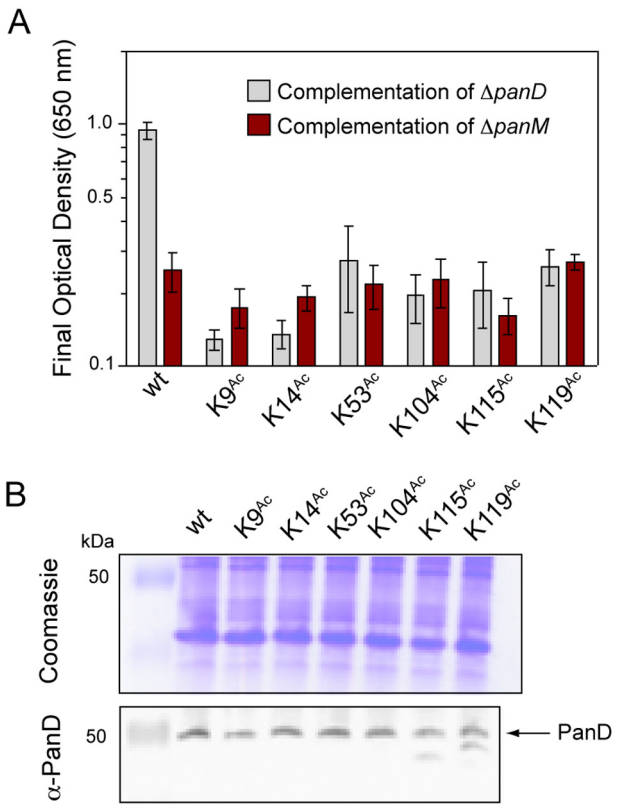

FIG 4 Complementation of Δ*panD* (JE13988; gray) and Δ*panM* (JE13226; red) *S. enterica* strains with *panD* genes encoding ^{Ac}K-pro-PanD variants. (A) Final optical densities of strains grown for 15 h on minimal glycerol medium lacking β-alanine. wt, wild type. (B) Western blot analysis of Δ*panM* strains grown in minimal glycerol medium containing β-alanine using rabbit polyclonal antibodies elicited against pro-PanD. All proteins in cell lysates were resolved under native PAGE conditions, with PanD migrating as a tetramer. Lane 1 contained a Precision Plus All Blue protein ladder (Bio-Rad).

pro-PanD proteins were synthesized under the growth conditions used, as shown by the results of Western blot analysis with anti-PanD rabbit polyclonal antibodies (Fig. 4B). Although some variants were produced at noticeably lower levels than wild-type pro-PanD, these levels still exceeded the native levels of PanD, which were below the level of detection by Western blot analysis (data not shown). All strains grew normally on minimal medium containing β-alanine (see Fig. S3 in the supplemental material), indicating that growth under our conditions was dependent on PanD activity. These results indicated that acetylated pro-PanD could not mature in the absence of PanM.

CoA binding is required for PanM activity *in vivo*. As mentioned above, acetyl-CoA stimulated PanM activity *in vitro*. We sought to determine whether binding of CoA was necessary for PanM activity *in vivo*. To study PanM-CoA interactions, CoA-binding was disrupted in PanM by changing residue Gly76 to a leucine. Gly76 (Fig. 1A) is part of the RGXG CoA-binding motif that is conserved in GNAT family members (11). Substitution of either glycine in this motif disrupted the ability to bind any form of CoA and disrupted acetyltransferase activity in other GNATs (14, 15). When expressed in *trans*, the *panM* allele encoding the PanM^{G76L} variant failed to restore growth of an *S. enterica* Δ*panM* strain in glycerol minimal medium lacking β-alanine (Fig. 5A). To allow detection of the PanM protein, *in vivo* experiments were performed using plasmids expressing a fusion protein of PanM and maltose-binding protein (MBP). The loss of *in vivo* activity for the PanM^{G76L} variant compared to results for wild-type PanM was not due to differences in the amount of translated protein. As shown in Fig. 5B, immunodetection of the MBP tag fused to PanM showed that both proteins were produced at similar levels under the growth conditions used. To verify that CoA binding had been disrupted by the G76L substitution, the wild-type PanM protein and PanM^{G76L} variant were purified (MBP tags removed), and their activities were measured in the presence or absence of acetyl-CoA. Unlike wild-type PanM, acetyl-CoA did not stimulate the activity of PanM^{G76L} (Fig. 5C). There is a small drop in PanD

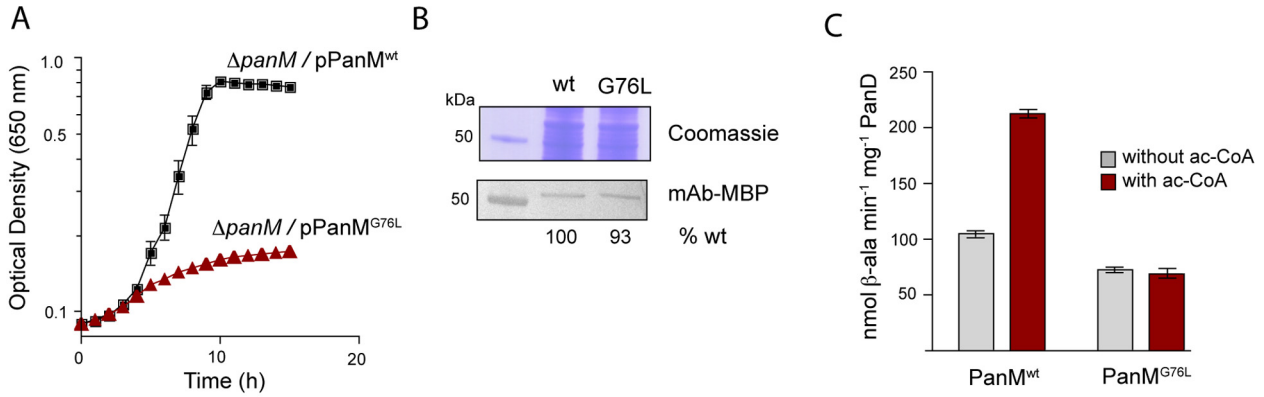

FIG 5 The CoA-binding-deficient PanM^{G76L} variant. (A) Complementation of Δ*panM S. enterica* (JE12555) in minimal glycerol medium with *panM* encoding either wild-type PanM (squares; black) or PanM^{G76L} (triangles; red). (B) Western blot analysis of strains from panel A grown in minimal glycerol medium containing β-alanine. PanM was detected using monoclonal antibodies raised against the maltose binding protein (MBP) tag fused to the N terminus of PanM. MBP-PanM bands were quantified using densitometry analysis and normalized to the wild-type sample. The SEM for two separate experiments was 5.9%. Lane 1 contained the Precision Plus All Blue protein ladder (Bio-Rad). (C) Specific activities of PanD after preincubation with either PanM^{wt} or PanM^{G76L}. Reactions were carried out in the absence of any CoA derivatives (gray) or with 100 μM acetyl-CoA (ac-CoA) (red).

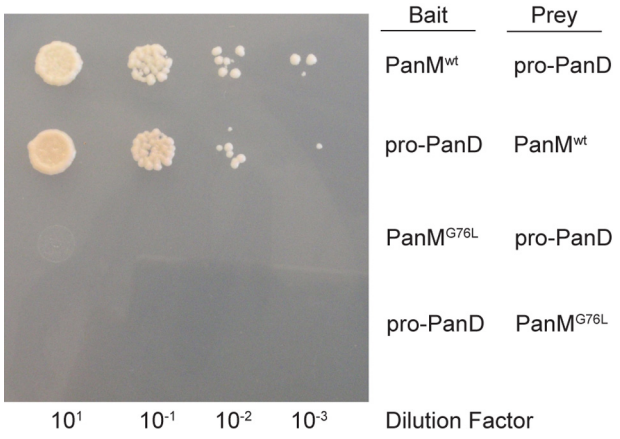

FIG 6 Yeast two-hybrid analysis. Growth of yeast two-hybrid reporter strain AH109 carrying bait and prey plasmids encoding proteins indicated to the right of the image. Overnight cultures of each strain were normalized to an OD_{600} of 0.1 and then serially diluted and spotted on SD medium lacking histidine.

activity when it is incubated with the PanM^{G76L} variant compared to that for incubation with wild-type PanM, regardless of the presence of acetyl-CoA. However, this activity is above the level of activity observed for PanD incubated without PanM (2), indicating that the PanM^{G76L} variant is active but cannot be stimulated by acetyl-CoA. These results indicated that CoA binding to PanM was necessary for PanM activity *in vivo*.

Disruption of CoA binding to PanM prevents PanM-PanD interactions. We employed the Matchmaker yeast two-hybrid system (Clontech) to determine whether acetyl-CoA binding to PanM was required for productive interactions with pro-PanD. The *panM* gene in bait and prey plasmids used in previous studies (2) was mutated to encode the G76L substitution. The Matchmaker reporter strain was transformed with the bait plasmid encoding pro-PanD and the prey plasmid encoding either PanM^{wt} or PanM^{G76L}. Strains containing the bait-prey swapped pairs were also constructed. Serial dilutions of all strains were spotted onto SD medium lacking histidine. In the Matchmaker system, histidine biosynthesis is controlled by the Gal4 transcription factor. The bait and prey plasmids each encode one domain of Gal4 fused to either pro-PanD or PanM. If pro-PanD and PanM interact, they bring together both domains of Gal4, allowing histidine biosynthesis to occur. The reporter strain grew in the absence of histidine when carrying a bait plasmid encoding wild-type PanM and a prey plasmid encoding pro-PanD (Fig. 6, row 1). The bait-prey swapped pair also grew (Fig. 6, row 2). When the strain carried either a bait or prey plasmid encoding PanM^{G76L}, the reporter strain did not grow (Fig. 6, rows 3 and 4) indicating that the CoA-binding deficient PanM^{G76L} did not bind to pro-PanD. Similar to results for other GNATs, these results revealed that CoA binding was necessary for PanM to interact with pro-PanD.

DISCUSSION

PanM is a GNAT homologue devoid of acetyltransferase activity but has acquired a new biological function. The Gcn5-like *N*-acetyltransferases (GNATs) are the most abundant family of acetyltransferases and are conserved in all domains of life (7). PanM is annotated as a GNAT on the basis of its primary sequence and its three-dimensional structure. However, PanM failed to transfer radiolabeled acetyl moieties to its partner pro-PanD (Fig. 2). Two other lines of evidence support the conclusion that PanM is not an acetyltransferase: (i) PanM lacks the conserved catalytic glutamate found in many enzymatically active GNATs (Fig. 1A), and (ii) no other charged residues in the vicinity of CoA are necessary for activity (Fig. 3). Although some loss of *in vivo* activity was observed for the PanM variant where all seven potential catalytic residues were replaced by alanine, we suspect the decrease in activity for this variant was due to protein folding or stability changes. This idea was supported by our attempts to purify the 7-substitution variant. The 5-substitution variant, which had wild-type levels of *in vivo* activity, was purified in milligram amounts with high purity. In contrast, purification of the 7-substitution PanM variant only yielded microgram levels of protein at <50% purity (see Fig. S4 in the supplemental material).

If acetylation of pro-PanD by PanM were the trigger for maturation, we would expect genetically acetylated pro-PanD to no longer require PanM for activation. Two results argue against this possibility: (i) acetylated pro-PanD failed to self-activate in the absence of PanM, and (ii) acetylation caused a loss of *in vivo* PanD activity, regardless of which lysine was modified (Fig. 4).

Collectively, our data indicate that PanM is a GNAT homologue that lacks acetyltransferase activity but has evolved the new function of triggering pro-PanD maturation. Interestingly, many other GNATs from diverse organisms appear to also lack the conserved catalytic glutamate (16). It is thus possible that, like PanM, some of these proteins may have evolved new functions, which may also require acetyl-CoA binding.

PanM is an acetyl-CoA sensor likely needed to respond to low free CoA levels. Although PanM lacks acetyltransferase activity, it has an acetyl-CoA binding motif (Fig. 1), and acetyl-CoA stimulates PanM activity *in vitro* (Fig. 2). In addition, CoA binding is necessary for its activity *in vivo* (Fig. 5) because the ability of PanM to bind CoA is necessary for PanM to interact with pro-PanD (Fig. 6). These data suggest that, like other GNATs, PanM may bind its substrates sequentially with acetyl-CoA binding first before pro-PanD can bind to it. Such binding kinetics would allow PanM to function effectively as an acetyl-CoA sensor. This idea is appealing since PanM regulates an early step in synthesis of the CoA precursor pantothenate (2). The levels of free CoA and acetyl-CoA often display an inverse relationship, because one form is produced at the expense of the other (9). That is, high levels of acetyl-CoA result in low levels of CoA, a situation that may signal the need for more CoA synthesis. PanM would be poised as a feed-forward regulator to sense high levels of acetyl-CoA and respond by activating more pro-PanD. It would be interesting to explore the *in vivo* levels of pro-PanD cleavage under various growth conditions, including both feast and famine conditions where CoA ratios would vary.

MATERIALS AND METHODS

Chemicals. Purification of ethyl-CoA is described in the supplemental material. [1-^{14}C]acetyl-CoA was purchased from Moravek Biochemicals. Protein concentrations were determined using the Bio-Rad protein assay dye reagent. All other chemicals were obtained from Sigma.

Strain construction. Strains used in this study are listed in Table S1 in the supplemental material. All *Salmonella* strains are derived from *S. enterica* serovar Typhimurium LT2. The DM10310 wild-type and DM285 *panD618::*Tn*10d*(Tc) strains were a gift from D. Downs (University of Wisconsin, Madison, WI). The JE13226 strain was constructed by resolv-

ing the kanamycin resistance cassette from the *panM*::*kan*$^+$ (JE12555) strain using established methods (17). The *ara-9 panD618*::Tn*10d*(Tc) strain (JE13988) was created by transduction using phage P22 HT105/1 *int-210* from DM285 into DM1310 (18, 19).

Plasmid construction. All plasmids used in this study are listed in Table S1 in the supplemental material, and primers used for plasmid construction are listed in Table S2. Plasmids encoding PanM and PanD variants were constructed using the QuikChange II site-directed mutagenesis kit (Agilent). The pPAN68 plasmid was used as a template to create the CoA-binding-deficient PanM^{G76L} variant. Potential PanM catalytic residues were mutated to alanines using the pPAN65 plasmid as the template. Plasmids expressing ^{Ac}K-pro-PanD were created by independently replacing each lysine codon in the *panD* gene of pPAN65 with amber stop codons. All mutations were verified by DNA sequencing using a BigDye kit (Applied Biosystems) at the Biotechnology Sequencing Center (University of Wisconsin, Madison, WI).

Bacterial growth conditions. Bacterial strains were grown in lysogeny broth (LB) (20, 21) or no-carbon E medium (NCE) (22) containing glycerol (20 mM) as the sole carbon and energy source. Ampicillin was added at 100 μg ml^{-1} and kanamycin and spectinomycin at 50 μg ml^{-1}, and β-alanine was used at 20 μM. All bacterial cultures were grown aerobically with shaking at 200 rpm, and growth was observed by the increase in optical density at 650 nm (OD_{650}) using an EL808 plate reader (Bio-Tek). Growth experiments were performed as biological triplicates and repeated at least twice. Statistical analyses were performed with data from all replicates. The genetically encoded acetyllysine tRNA system described elsewhere (23) was used to produce ^{Ac}K-pro-PanD variants. The pCDF PylT-1 and pAcKRS-3 plasmids, encoding *Methanosarcina barkeri* pyrrolysyl-tRNA synthetase and the amber suppressor tRNA^{CUA}, were transformed into the Δ*panM* (JE13226) and Δ*panD* (JE13988) *S. enterica* strains. Each resultant strain was transformed with a plasmid expressing a *panD* gene where a lysine codon was mutated to an amber stop codon. Strains were grown in NCE glycerol medium containing ampicillin, kanamycin, spectinomycin, 2 mM acetyllysine, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 0.5% Casamino Acids (vitamin assay quality).

Yeast growth conditions. Yeast strains were grown aerobically at 30°C on either synthetic minimal (SD) medium or yeast extract peptone dextrose (YPD) medium (24). The Matchmaker AH109 yeast reporter strain was transformed with bait and prey plasmids, which were maintained by auxotrophic selection on SD medium devoid of leucine and tryptophan. Overnight (5-ml) cultures were normalized to an optical density (at 600 nm) of 0.1 then serially diluted. Samples (4 μl) of each dilution were spotted onto SD plates lacking histidine to test for bait-prey interactions. Plates were imaged using a Fotodyne digital imaging system after 60 h of growth.

Primary sequence alignments. Motif A of *Tetrahymena* GCN5 and human PCAF were obtained from the literature (12, 13). Motif A of *S. enterica* PanM was determined from an alignment with *E. coli* PanM using the software program ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2), based on the *E. coli* PanM motif A established by others (16). The motif A sequences from each protein were then aligned using ClustalW.

L-Aspartate decarboxylase (PanD) activity assays. PanD activity assays were performed as described elsewhere (2) in two steps: (i) preincubation with PanM to allow maturation of pro-PanD and (ii) PanD activity assays started by the addition of the substrate L-aspartate. Reaction mixtures contained pro-PanD (3 μg) preincubated with 0.6 μg PanM (pro-PanD to PanM ratio of 5:1). CoA derivatives were added to the preincubation mixture at a final concentration of 100 μM. Results were graphed, and two-tailed *t* tests were performed using the software program Prism v4.0 (GraphPad).

PanM Western blot analysis. The Δ*panM* strain (JE12555) was transformed with pPAN68 (encoding H_6-MBP-PanM) or pPAN82 (encoding H_6-MBP-PanM^{G76L}) and grown to late log phase (OD_{600} of ~0.8) in minimal glycerol medium containing β-alanine. Cells were pelleted by centrifugation at 5,000 × *g* for 15 min and resuspended in 0.5 ml of HEPES buffer (50 mM, pH 7). Cells were disrupted by sonication (30 s on setting 5) using a 550 sonic dismembrator (Fisher Scientific) and clarified by centrifugation at 14,000 × *g*. Samples (25 μg) of each lysate were boiled for 10 min and then resolved on a denaturing 12% acrylamide gel (25). Duplicate samples were either stained with Coomassie brilliant blue to visualize total protein or transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore). H_6-MBP-PanM was detected using mouse anti-MBP monoclonal primary antibodies (Invitrogen) and goat anti-mouse immunoglobulin G alkaline phosphatase (AP)-conjugated secondary antibodies. PanM was visualized using the AP substrate nitroblue tetrazolium--5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) (Thermo, Fisher). Western blots were imaged using a Fotodyne imaging system and analyzed by densitometry using TotalLab v2005 software (Nonlinear Dynamics). Statistical analyses were performed with data from three independent experiments using Prism v4.0 (GraphPad).

Purification of PanM^{G76L}. The pPAN82 plasmid was transformed into *E. coli* C41 (DE3) and purified by nickel affinity chromatography as described elsewhere for wild-type PanM (2). PanM^{G76L} was expressed as a fusion protein with H_6-MBP, which was removed by tobacco etch virus protease cleavage, allowing purification of untagged PanM^{G76L}.

Protein acetylation assays. Protein acetylation assays were performed as described elsewhere (26) with the following modifications. Reaction mixtures (30 μl) containing 6 μg (each) of PanM and pro-PanD and [1-^{14}C]acetyl-CoA (100 μM) in HEPES buffer (50 mM, pH 7) were incubated at 37°C for 2 h. Reactions were stopped by mixing with an equal volume of Laemmli sample buffer (Bio-Rad) and boiling for 10 min. Samples (40 μl; 4 μg PanD) were loaded onto a Mini-Protean TGX 4 to 20% acrylamide gel (Bio-Rad) and a Mini-Protean Any kD TGX gel (Bio-Rad). Proteins were visualized by Coomassie blue staining, and acetylation (the presence of [^{14}C]acetyl moieties) was visualized using an FLA9000 phosphorimager (GE Healthcare). The brightness and contrast of gel images were adjusted in the software program Photoshop vCS2.

PanD Western blot analysis. The Δ*panM* (JE13226) strain carrying pCDF PylT-1, pAcKRS-3, and either pPAN24, pPAN29, pPAN30, pPAN31, pPAN47, or pPAN48 were grown as described above in minimal glycerol medium containing ampicillin, kanamycin, spectinomycin, IPTG, Casamino Acids, and β-alanine to mid-log phase (OD_{600} of ~0.6). Lysates were prepared as described above for PanM Western blots. Samples (25 μg total protein) of each lysate were separated on a nondenaturing 12% acrylamide gel (25). Western blots were developed and analyzed as described for PanM Western blots with the exception of the primary antibody: rabbit polyclonal antibodies raised against pro-PanD (Harlan).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00158-12/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.3 MB.
Figure S4, PDF file, 0.2 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0 MB.

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We do not have any conflicts of interest to declare.

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