

Comparative Study of Eis-like Enzymes from Pathogenic and Nonpathogenic Bacteria

Keith D. Green,[†] Rachel E. Pricer,[‡] Megan N. Stewart,[#] and Sylvie Garneau-Tsodikova^{*,†}

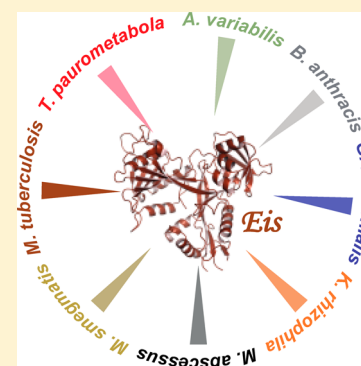
[†]College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0596, United States

[‡]Chemical Biology Doctoral Program and [#]Department of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States

Supporting Information

ABSTRACT: Antibiotic resistance is a growing problem worldwide. Of particular importance is the resistance of *Mycobacterium tuberculosis* (*Mtb*) to currently available antibiotics used in the treatment of infected patients. Up-regulation of an aminoglycoside (AG) acetyltransferase, the enhanced intracellular survival (Eis) protein of *Mtb* (Eis_*Mtb*), is responsible for resistance to the second-line injectable drug kanamycin A in a number of *Mtb* clinical isolates. This acetyltransferase is known to modify AGs, not at a single position, as usual for this type of enzyme, but at multiple amine sites. We identified, using in silico techniques, 22 homologues from a wide variety of bacteria, that we then cloned, purified, and biochemically studied. From the selected Eis homologues, 7 showed the ability to modify AGs to various degrees and displayed both similarities and differences when compared to Eis_*Mtb*. In addition, an inhibitor proved to be active against all homologues tested. Our findings show that this family of acetyltransferase enzymes exists in both mycobacteria and non-mycobacteria and in both pathogenic and nonpathogenic species. The bacterial strains described herein should be monitored for rising resistance rates to AGs.

KEYWORDS: acetylation, aminoglycoside acetyltransferases, enzyme inhibitors, *n*-propionylation, sequential reactions



Resistance to aminoglycosides (AGs, Figure 1) has been a growing problem with numerous species of bacteria, driving the development of novel antibiotics and research into the causes of resistance. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, all together known as the “ESKAPE” pathogens, have been particularly difficult to treat.¹ The most common mode of resistance to AGs is their inactivation by AG-modifying enzymes (AMEs), such as the AG acetyltransferases (AACs), AG phosphotransferases (APHs), and AG nucleotidyltransferases (ANTs). AACs, comprising the majority of these AMEs, are known for their monoacetylating specificities toward the 2′-, 3-, or 6′-positions of a variety of AGs. More recently, a unique AAC, the enhanced intracellular survival (Eis) protein of *Mycobacterium tuberculosis* (*Mtb*), was found capable of multiacetylating AGs.^{2,3} It was originally shown that it is the up-regulation of the *eis* gene that is responsible for resistance to kanamycin A (KAN) in a large number of clinical isolates,⁴ and since then, numerous additional papers on mutation in the *eis* promoter have been published.^{5–7}

We have shown that multiacetylation by Eis completely inactivates AGs.^{8,9} We have also performed studies monitoring the effect that different modifications have on a second modification and its catalyzing enzyme.⁹ We found that Eis from *Mtb* (Eis_*Mtb*) is capable of multiacetylating AGs,² and we observed a similar multiacetylating behavior when studying Eis from *Mycobacterium smegmatis* (Eis_*Msm1*),¹⁰ Eis from

Anabaena variabilis (Eis_*Ava*),¹¹ and the Eis homologue from *Bacillus anthracis* (Eis_*Ban*).³⁷ AACs are known to be cosubstrate promiscuous,⁸ although we have shown that Eis_*Mtb* has a much more limited cosubstrate tolerance.¹² In addition, we have studied the metabolically possible competition between acetyl-CoA (AcCoA) and *n*-propionyl-CoA (ProCoA) and the effects it has on multiacetylation of AGs.

Although the cellular role of Eis is still unclear, there have been several proposed functions and substrates for this enzyme. Eis_*Mtb* has been found in the sera of infected patients, suggesting it may play a role in the persistence of bacterial infection.¹³ The *eis* protein product that is secreted from the organism has been proposed to modulate the host inflammatory response.¹⁴ Additionally, T-cells treated with Eis_*Mtb* have an inhibited ERK1/2, JAK pathway, which subsequently produces TNF- α and IL-4, thus disrupting the cross-regulation of T-cells.¹⁵ This effect of aiding the survival of the *Mtb* bacteria in macrophages is thought to be associated with Eis_*Mtb*'s extreme stability under harsh conditions.¹⁶ Suh and co-workers have shown that this modulation of the host immune system may happen through the acetylation of DUSP16/MKP-7 by Eis_*Mtb*¹⁷ and have performed docking studies that corroborate this hypothesis.¹⁸

In an effort to better understand Eis's role in bacteria and attempt to understand the evolution that occurred with this

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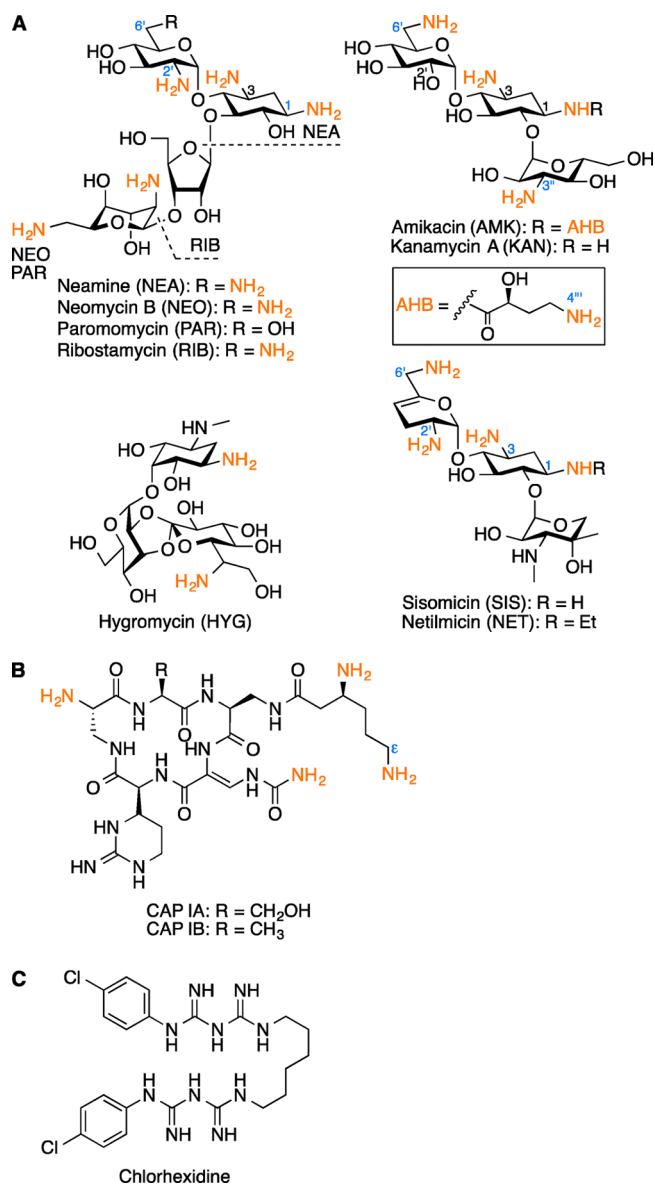


Figure 1. Chemical structures of (A) all AGs found to be modified by Eis homologues in this study, (B) the polypeptide capreomycin (sold as a mixture of CAP IA and CAP IB), and (C) an Eis_ *Mtb* inhibitor, chlorhexidine.

gene in *Mtb* to modify AGs along with its other substrates, we looked to other bacterial species containing Eis homologues. Through a rigorous *in silico* search, we found Eis homologues in a variety of mycobacteria and non-mycobacteria (Figure 2). Herein, we examine several properties of these enzymes and compare them to Eis_ *Mtb*. We present *in silico* results for bacterial species containing Eis homologues. We cloned and purified 18 new Eis homologues. We tested these proteins against a small group of antibacterial compounds checking for any acetylation activity. For each AG-active enzyme, we determined the kinetic parameters for a representative of each structural class of AG, kanamycin (KAN) for 4,6-disubstituted deoxystreptamine (DOS) and PAR for 4,5-disubstituted DOS. For combinations producing a positive UV-vis test, we determined the degrees of acetylation and *n*-propionylation. We also explored the cosubstrate tolerance of AG-active enzymes and examined the competition of AcCoA

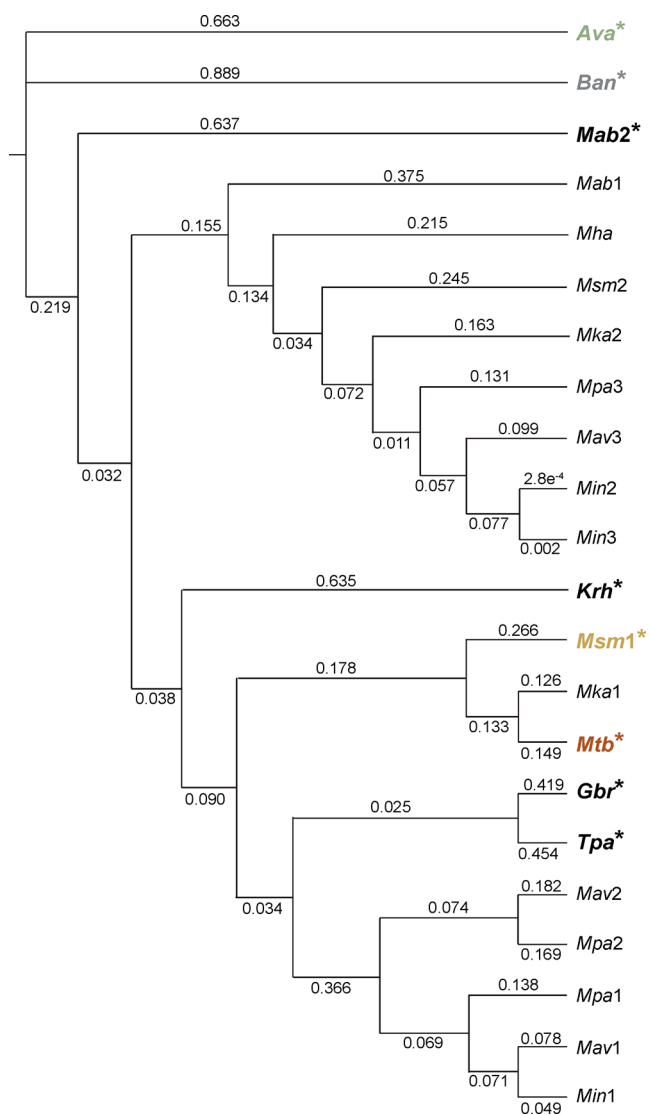


Figure 2. Phylogenetic tree analysis of the 22 Eis homologues studied based on their amino acid sequences. The numbers are proportional to the distance of the evolutionary branches of the tree. The abbreviated names of the tree correspond to the following species and gene: *Anabaena variabilis* ATCC 29413 (Ava); *Bacillus anthracis* 34F2 Sterne strain (Ban); *Gordonia bronchialis* ATCC 25592 (Gbr); *Kocuria rhizophila* ATCC 9341 (Krh); *Mycobacterium abscessus* ATCC 19977 genes MAB4124 (Mab1) and MAB4532c (Mab2); *Mycobacterium avium* subsp. *avium* ATCC 25291 genes MaviaA2_07418 (Mav1), MaviaA2_15865 (Mav2), and MaviaA2_00691 (Mav3); *Mycobacterium hassiacum* DSM 44199 (Mha); *Mycobacterium intracellulare* ATCC 13950 genes MintA_24600 (Min1), MintA_21739 (Min2), and OCU_01560 (Min3); *Mycobacterium kansasii* ATCC 12478 genes Mkan A1_16037 (Mka1) and Mkan A1_05800 (Mka2); *Mycobacterium parascrofulaceum* ATCC BAA-614 genes ZP_06852004 (Mpa1), ZP_06848356 (Mpa2), and ZP_06849426 (Mpa3); *Mycobacterium smegmatis* MC2 155 genes MSMEG_3513 (Msm1) and MSMEG_4540 (Msm2); *Mycobacterium tuberculosis* H37Rv (Mtb); *Tsukamurella paurometabola* ATCC 8368 (Tpa). Active enzymes are indicated with an asterisk. The percent identity of each homologue compared to that of Eis_ *Mtb* is presented in the Supporting Information (Table S1).

and ProCoA binding in the GNAT domain. In an effort to identify broad or specific inhibitors of these enzymes, a

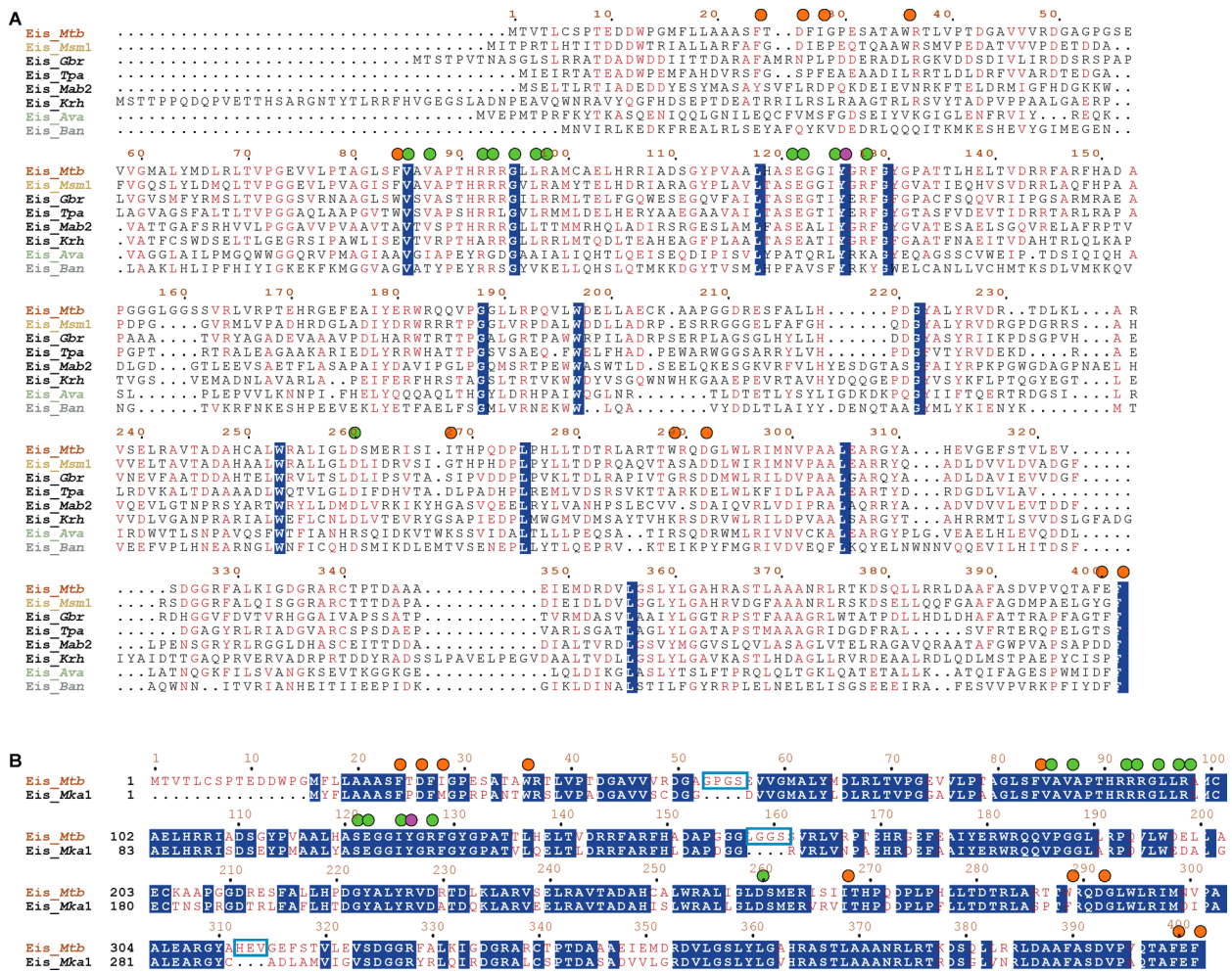


Figure 3. (A) Alignment of all Eis homologue protein sequences found to be active in acetylating AGs in this work. (B) Alignment of *Eis_Mtb* and its closest homologue in amino acid sequence tested, *Eis_Mka1*. Turquoise boxes indicate areas of difference for the *Mka1* homologue, which are hypothesized to be important in protein–protein interactions of the Eis monomers. For both alignments, the catalytic tyrosine (Y) residue is indicated with a purple circle, AG-binding site residues are indicated with orange circles, and AcCoA-binding site residues are indicated with green circles. All of the residues marked with colored circles were originally identified by site-directed mutagenesis of the *Eis_Mtb* enzyme.^{2,36} White letters with blue background indicate amino acids completely conserved throughout the sequences, whereas red residues indicate amino acids highly conserved throughout the sequences.

compound known to inhibit *Eis_Mtb*¹⁹ was tested against the AG-active homologues.

RESULTS AND DISCUSSION

In Silico Analysis of Potential Eis Homologues. A BLAST search against *Eis_Mtb* revealed a myriad of Eis homologues. Eis proteins were found in the genome of several mycobacteria including *Mycobacterium avium* subsp. *avium* ATCC 25291 (*Mav*), *Mycobacterium kansasii* ATCC 12478 (*Mka*), *Mycobacterium abscessus* ATCC 19977 (*Mab*), *Mycobacterium hassiacum* DSM 44199 (*Mha*), *Mycobacterium intracellulare* ATCC 13950 (*Min*), and *Mycobacterium parascrofulaceum* ATCC BAA-614 (*Mpa*). Because Eis was originally found in *Mtb*²⁰ and later in *Msm*,¹⁰ it is not surprising Eis homologues were found in other mycobacteria. Curiously, some species were found to have two (*Mab* and *Mka*) or three (*Mav*, *Min*, and *Mpa*) Eis homologues, and upon further examination of the genomic sequence, a second Eis-like protein was found in *Msm*. Many of the listed mycobacteria are involved in atypical mycobacterial infections,²¹ and *Mab* has been noted, particularly, as being “a new antibiotic night-

mare”;²² however, this is likely due to mutations in the rRNA,²³ yet drug combinations are being investigated for this pathogenic species.²⁴ *Mav*–*Min* complex, *Mka*, and *M. fortuitum* extracts have been analyzed for AMEs, and an unspecified AAC was found to act on KAN, tobramycin (TOB), and neomycin B (NEO) in *Mka* and *M. fortuitum*.²⁵ Outside the mycobacterial genus, three other species were found to harbor Eis homologues in their respective genomes: *Gordonia bronchialis* ATCC 25592 (*Gbr*), *Kocuria rhizophila* ATCC 9341 (*Krh*), and *Tsakumurella paurometabola* ATCC 8368 (*Tpa*). As the name suggests, *Gbr* is a bacterium that often affects the lungs; the sample used here was originally isolated from the sputum of a patient with cavitary pulmonary disease. *Tpa* was originally isolated from the microflora of a bedbug,²⁶ and *Krh* is a soil microorganism,²⁷ but is now appearing more often in the clinic.^{28,29} A phylogenetic tree (Figure 2) of these proteins was constructed, revealing that these enzymes break into two major groups and three outliers (*Eis_Ava*, *Eis_Ban*, and *Eis_Mab2*). One of the groups consists of strictly proteins from mycobacteria that align well (35% exact amino acid match) with each other and include *Eis_Mab1*,

Table 1. Comparison of the Number of Modifications for Reactions of Eis Homologues with Various AGs and AcCoA or ProCoA^a

AG	AcCoA							
	Eis_Ava ^b	Eis_Ban ^c	Eis_Gbr	Eis_Krh	Eis_Mab	Eis_Msm1 ^{b,d}	Eis_Mtb ^{b,c,d,e}	Eis_Tpa
AMK	tri	di	di	di	×	tri	tri	tri
HYG	×	di	mono	mono	mono	mono	di	di
KAN	mono	di	mono	di	di	di	di	di
NEA	tri	tri	tri	tri	tri	tri	tri	tri
NEO	tri	tri	tri	tetra	tetra	tri	tri	tetra
NET	di	mono	mono	di	di	di	di	di
PAR	tri	tri	tri	tri	tri	tri	di	tri
RIB	tri	tri	di	di	di	tri	tri	di
SIS	di	di	di	tri	di	tri	tri	di
AG	ProCoA							
	Eis_Ava	Eis_Ban	Eis_Gbr	Eis_Krh	Eis_Mab	Eis_Msm1	Eis_Mtb ^e	Eis_Tpa
AMK	mono	di	di	mono	×	di	di	di
HYG	×	di	mono	mono	mono	mono	mono	mono
KAN	mono	mono	mono	mono	di	di	di	di
NEA	di	tri	di	di	tri	di	di	tri
NEO	di	tri	tri	di	di	tri	tri	di
NET	mono	mono	mono	mono	di	mono	mono	di
PAR	di	di	di	di	tri	di	mono	tri
RIB	di	tri	di	di	di	di	di	di
SIS	di	di	di	di	di	mono	mono	di

^a× indicates that the AG is not a substrate for the enzyme tested. ^bData from ref 11. ^cData from ref 37. ^dData from ref 10. ^eData from ref 12.

Eis_Mav3, Eis_Mha, Eis_Min2, Eis_Min3, Eis_Mka2, Eis_Msm2, and Eis_Mpa3. The second group contains all of the remaining Eis homologues that align poorly (7% exact amino acid match). Taking a closer look at their amino acid sequences, we found most homologues to have the AcCoA binding residues and the catalytic residues in the appropriate position. However, the residues proposed to bind the AG are found to a lesser extent in the homologous enzymes. An amino acid sequence alignment of all 22 Eis homologues is presented in Figure S1 in the Supporting Information, and sequence alignments for AG active and inactive Eis homologues are presented in Figures 3A and S2, respectively.

AG Activity of Eis Enzymes. All Eis homologues were tested against a small library of antibiotic compounds including amikacin (AMK), chloramphenicol (CAM), capreomycin (CAP), ciprofloxacin, hygromycin (HYG), KAN, neamine (NEA), NEO, netilmicin (NET), norfloxacin, paromomycin (PAR), ribostamycin (RIB), sisomicin (SIS), spectinomycin (SPT), and streptomycin (STR) to establish the substrate profile of these enzymes (Table 1). CAM was included in our study because there are known CAM-modifying enzymes;³⁰ CAP was included because of the recent discovery that Eis_Mtb can modify this cyclic peptide;³¹ and the fluoroquinolones were included as an AAC mutant was found to acetylate these drugs.³² Enzymes purified from Mav, Mka, Min, and Mpa, along with one enzyme from Mab (Eis_Mab1) and the new Msm enzyme (Eis_Msm2), were all found to be AG inactive. These enzymes also all proved to be less soluble than the AG active homologues, with Eis_Mka2 being completely insoluble and remaining in the pelleted cell debris under the conditions used to purify Eis_Mtb. Eis_Mha had excellent expression and solubility, but was also AG inactive under conditions identical to those used in acetylating assays with Eis_Mtb. Eis_Gbr, Eis_Krh, Eis_Mab2, and Eis_Tpa all showed various degrees of activity with the AGs tested (AMK, HYG, KAN, NEA, NEO,

NET, PAR, RIB, and SIS) with the exception of Eis_Mab2, which proved to be inactive when tested with AMK. Although the explanation of inactivity of Eis_Mab2 toward AMK would be highly speculative without a crystal structure, the 14 Eis homologues showing complete AG inactivity can be analyzed on the basis of their amino acid sequence. Eis_Mab1, Eis_Mav3, Eis_Mha, Eis_Min2, Eis_Min3, Eis_Mka2, Eis_Msm2, and Eis_Mpa3 all find themselves in one main branch of the phylogenetic tree (Figure 2). It is highly possible that they have some other unknown biological purpose that differs from that of Eis_Mtb. One residue that is found to differ from Eis_Mtb in all of these AG-inactive Eis homologues is F²⁴ (Eis_Mtb numbering used throughout). In the majority of these homologues, an arginine residue is found in place of F²⁴ (Figure S2). This arginine would repel AGs under physiological conditions. The remaining inactive enzymes are in the second branch of the phylogenetic tree and include the AG-active homologues Eis_Mtb, Eis_Msm1, Eis_Gbr, Eis_Krh, and Eis_Tpa. Whereas the homologues from the first branch were missing an important AG binding residue, Eis_Mav1, Eis_Mav2, Eis_Min1, Eis_Mpa1, and Eis_Mpa2 are all missing one residue for AcCoA binding, R¹²⁸, which is replaced by either glycine or isoleucine. This lack of charge at this position may not allow AcCoA to bind with affinity similar to that with Eis_Mtb (Figure S2). Most surprising is the AG inactivity of Eis_Mka1, which has 74% homology to Eis_Mtb. The only explanation we can propose from the sequence alignment is that Eis_Mka1 is 26 amino acids shorter and is missing amino acid sequences from G⁵² to S⁵⁵, from L¹⁵⁸ to S¹⁶¹, and from H³¹² to V³¹⁴. Upon examination of the Eis_Mtb crystal structure (PDB ID 3R1K)² these residues form three sites for protein–protein interaction, possibly responsible for oligomerization (Figure 3B) as each missing sequence is in an area where protein–protein interactions are likely to be. This might indicate that Eis_Mka does not form hexamers. It has been

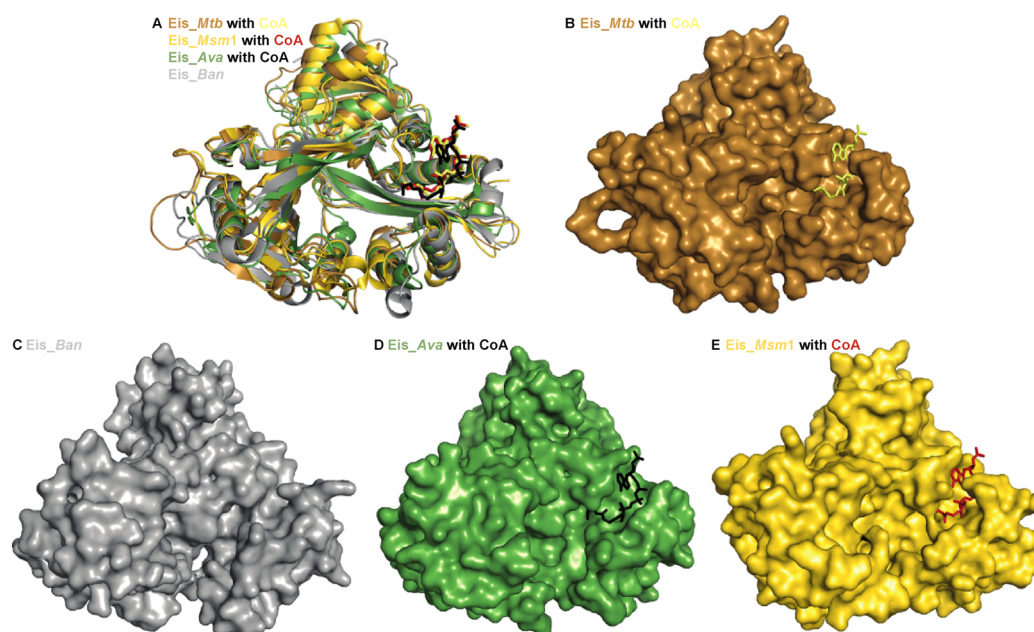


Figure 4. Comparison of crystal structures of *Eis_Mtb* (PDB 3R1K, 1.95 Å),² *Eis_Msm1* (PDB 3SXN, 2.03 Å),¹⁷ *Eis_Ava* (PDB 2OZG, 2.00 Å),¹¹ and *Eis_Ban* (PDB 3N7Z, 2.75 Å). (Only one monomer of these hexameric structures is shown for clarity.) Panel A depicts the overlay of one monomer from each of the four structures and shows that there is little variation in the overall structure of this family of enzymes. Surface cartoons shown in panels B–E demonstrate subtle differences in the active sites of the Eis homologues from (B) *Mycobacterium tuberculosis* (shown in brown with CoA as yellow stick), (C) *Bacillus anthracis* (shown in gray in the absence of CoA), (D) *Anabaena variabilis* (shown in green with CoA as black stick), and (E) *Mycobacterium smegmatis* (shown in gold with CoA as red stick).

shown that the *Eis_Mtb* hexameric form is required for activity.¹⁶ It is known that the *Eis_Mtb* hexamer is extremely resilient to a variety of conditions,¹⁶ and based on what we can hypothesize from the *Eis_Mka1* data, this area could be heavily involved in activity, despite its remote location in relationship to the enzyme active site. Crystal structures of all enzymes would allow for a better understanding of inactivity.

There are structures available for three *Eis_Mtb* homologues, *Eis_Ava*, *Eis_Ban*, and *Eis_Msm1*, which have been previously published with a comparative structural analysis.^{2,3,10,11,33,37} Shown in Figure 4 are the four currently known crystal structures of *Eis_Mtb* and its three crystallized homologues. The cartoon overlay of the four crystal structures (Figure 4A) shows only minor changes in the backbone of the structure with only one or two misaligned α -helices and a few disordered loops. The differences are significantly more apparent when looking at the space-filled models (Figure 4B–E), where the AG-binding site (left of the AcCoA-binding site in Figure 4B,D,E) varies. The variance in the AG-binding site is likely why each enzyme reacts with the same AGs differently. Recent work has shown that the AG TOB can bind to *Eis_Mtb* in two distinct orientations, placing two amino groups in the appropriate configuration for acetylation at the 6'- and 3"-positions.³ This emphasizes the fact that the AG-binding site can dramatically affect not only the identity of AGs that can bind to the Eis enzymes but also the various orientations that any given AG can adopt in the AG-binding pocket.

Acetylation of the Polypeptide Antibiotic CAP. We recently showed that in addition to modifying AGs, *Eis_Mtb* is also capable of acetylating the nonribosomal peptide CAP.³¹ In addition to the panel of AGs (Figure 1A), we also tested the seven homologues able to acetylate AGs for their ability to modify CAP. Just as we observed for the AGs, there were several differences within this family of enzymes. *Eis_Krh*,

Eis_Mab, *Eis_Msm*, *Eis_Mtb*, and *Eis_Tpa* all had very similar initial rates in the UV–vis assay, when CAP was used as the substrate (Figure 5). This suggests that these enzymes all

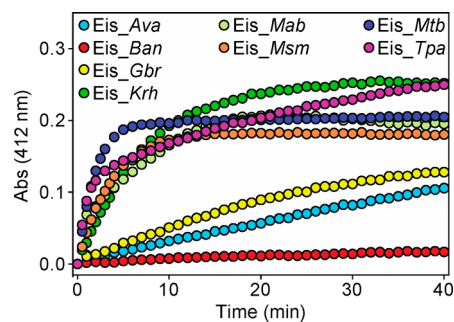


Figure 5. UV–vis curves monitoring the acetylation of CAP by various Eis homologues.

catalyze the modification of CAP similarly. *Eis_Ava* and *Eis_Gbr* both showed significantly reduced reactions rates when modifying CAP, suggesting that these two homologues bind CAP less effectively than the previous five or have a lower catalytic efficiency. Interestingly, *Eis_Ban* did not react with CAP at all. These data supplement the AG data showing that the differences observed in the crystal structures of Eis proteins (Figure 4) can significantly alter the ability of said enzymes to modify different amino groups.

Kinetic Parameters of AG-Active Eis Homologues for KAN and PAR. Kinetic parameters were determined for *Eis_Gbr*, *Eis_Krh*, *Eis_Mab2*, and *Eis_Tpa* and compared to those previously published for *Eis_Ava*,¹¹ *Eis_Ban*,³⁷ *Eis_Msm1*,¹⁰ and *Eis_Mtb*.² Kinetic values are presented in Table 2 along with a comparison of the catalytic turnover (k_{cat}) and catalytic efficiency (k_{cat}/K_m) to *Eis_Mtb*. KAN bound with

Table 2. Kinetic Parameters Determined for Eis Homologues from Various Species by Using AcCoA with different AGs

Eis homologue	AG	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	$k_{\text{cat,Xxx}}/k_{\text{cat,Mtb}}$	$k_{\text{cat}}/K_m\text{Xxx}/k_{\text{cat}}/K_m\text{Mtb}$
Eis_Ava ^a	KAN	1002 ± 43	0.205 ± 0.004	205 ± 9	0.39	0.13
	PAR	237 ± 36	0.183 ± 0.013	773 ± 132	1.35	0.48
Eis_Ban ^b	KAN	57 ± 9	0.135 ± 0.008	2368 ± 399	0.26	1.49
	PAR	44 ± 11	0.019 ± 0.001	432 ± 110	0.14	0.35
Eis_Gbr	KAN	1020 ± 350	0.672 ± 0.036	659 ± 229	1.28	0.41
	PAR	763 ± 150	2.05 ± 0.18	2687 ± 579	15.07	2.17
Eis_Krh	KAN	268 ± 47	0.042 ± 0.002	157 ± 28	0.08	0.10
	PAR	435 ± 52	1.68 ± 0.07	3862 ± 406	12.35	3.12
Eis_Mab2	KAN	125 ± 27	0.124 ± 0.007	992 ± 377	0.24	0.62
	PAR	144 ± 14	2.83 ± 0.07	19653 ± 1972	20.80	15.90
Eis_Msm1 ^{a,c}	KAN	665 ± 42	0.360 ± 0.010	541 ± 37	0.68	0.34
	PAR	738 ± 158	0.235 ± 0.033	318 ± 82	1.73	0.26
Eis_Mtb ^{a,c}	KAN	330 ± 40	0.526 ± 0.027	1594 ± 250	1.00	1.00
	PAR	110 ± 21	0.136 ± 0.012	1236 ± 260	1.00	1.00
Eis_Tpa	KAN	35 ± 8	0.163 ± 0.007	4657 ± 1083	0.31	2.92
	PAR	180 ± 10	5.92 ± 0.09	32889 ± 1894	43.53	26.60

^aData from ref 11. ^bData from ref 37. ^cData from ref 36.

the highest affinity to Eis_Tpa, binding 9-fold better than Eis_Mtb. Meanwhile, PAR displayed 2.5-fold tighter binding to Eis_Ban over Eis_Mtb. KAN reacted at a rate of around 0.7 reaction per second with Eis_Gbr, 1.28 times faster than Eis_Mtb, whereas PAR had the quickest turnover time with Eis_Tpa at 5.92 reactions per second, ~44-fold faster than Eis_Mtb. The most efficient combinations were with AGs and Eis_Tpa, showing ~3- and ~27-fold increases in efficiency for KAN and PAR when compared to Eis_Mtb, respectively. Additionally, Eis_Krh showed poor kinetic parameters for KAN and heightened parameters for PAR. This is somewhat surprising considering that Eis_Krh is roughly 10-kDa larger than Eis_Mtb, with two extraneous peptide chains throughout the amino acid sequence (Figure 3A). Although an in-depth kinetic study was not performed for each enzyme, there are no data that indicate that these enzymes do not react similarly to Eis_Mtb, having a random sequential mechanism.³⁴ Whereas Eis_Mtb may have evolved to modify AGs due to selective pressure, it seems that the homologues from Gbr and Tpa have a greater affinity for AGs and these species should be watched for development of AG resistance.

Number of Acetylations. To investigate the extent to which the AG-active Eis homologues can acetylate AGs, we determined the number of sites acetylated by these enzymes on AMK, HYG, KAN, NEA, NEO, NET, PAR, RIB, and SIS (Table 1 and Figures S11, S15, S19, and S26). All Eis homologues were found to acetylate NEA three times. HYG, KAN, and NET all showed mono- or diacetylation by the enzymes, whereas AMK, PAR, RIB, and SIS were observed to be either di- or triacetylated. Interestingly, NEO was acetylated between two and four times. This tetra modification was observed previously only for Eis_Mtb and Eis_Msm1 with TOB. Only Eis_Krh, Eis_Mab, and Eis_Tpa were found to tetra-acetylate NEO. This suggests that the binding pockets or at least the AG-binding portion of these enzymes are more

flexible in their substrate binding. Eis_Gbr showed the lowest number of acetylations, in total having monoacetylated three of the AGs tested (HYG, KAN, and NET), suggesting a more limited enzyme–AG interaction. The only other mycobacterial enzyme, Eis_Mab, showed similar patterns of acetylation to those of Eis_Mtb and Eis_Msm1, but was more limited in its substrate profile, not accepting AMK and only diacetylating RIB.

Cosubstrate Tolerance of Eis Enzymes. The AG-active Eis homologues were tested with a variety of acyl-CoAs to establish their cosubstrate promiscuity (Table 3). As we have previously shown, Eis_Mtb had a very limited cosubstrate tolerance, transferring acyl groups from AcCoA, ProCoA, crotonyl-CoA, and malonyl-CoA only.¹² In addition to the four acyl-CoAs used by Eis_Mtb, *n*-butyryl-CoA, *n*-hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, and *i*-valeryl-CoA were tested as potential cosubstrates. All enzymes could only transfer acyl groups from AcCoA and ProCoA, and Eis_Krh could utilize malonyl-CoA to a lesser extent. These data confirm that despite the poor sequence alignment, the CoA-binding pocket of the Eis homologues studied is very likely similar to that of Eis_Mtb.

Degree of *n*-Propionylation. The number of *n*-propionylations each enzyme catalyzes with the different AGs was also analyzed to glean more insight into the size of the catalytic pocket (Tables 1 and S3–S9 and Figures S5, S8, S12, S16, S20, S23, and S27). ProCoA, being slightly larger than AcCoA, is not likely to be used as efficiently or result in as many modifications. As expected, all *n*-propionylations were of equal number or less than the number of acetylations observed for the Eis homologue–AG combinations tested. *n*-Propionylation was equal to acetylation for Eis_Mab2 in all cases, for seven of nine combinations using Eis_Ban (all except KAN and PAR) and Eis_Gbr (all except NEA and PAR), and six combinations for Eis_Tpa (all except AMK, KAN, and

Table 3. Comparison of the Cosubstrate Tolerance for Eis Homologues from Various Species Using NEO as the Substrate^a

acyl-CoA	Eis_Ava ^b	Eis_Ban	Eis_Gbr	Eis_Krh	Eis_Mab	Eis_Msm1	Eis_Mtb ^c	Eis_Tpa
Ac	✓	✓	✓	✓	✓	✓	✓	✓
Pro	✓	✓	✓	✓	✓	✓	✓	✓
<i>n</i> -butanoyl	×	×	×	×	×	×	×	×
<i>n</i> -hexanoyl	×	×	×	×	×	×	×	×
octanoyl	×	×	×	×	×	×	×	×
decanoyl	×	×	×	×	×	×	×	×
lauroyl	×	×	×	×	×	×	×	×
myristoyl	×	×	×	×	×	×	×	×
palmitoyl	×	×	×	×	×	×	×	×
crotonyl	×	×	×	×	×	×	✓	×
malonyl	×	×	×	✓	×	×	✓	×
<i>i</i> -valeryl	×	×	×	×	×	×	×	×

^a× indicates that the acyl-CoA is not a cosubstrate of the Eis enzyme tested; ✓ indicates that the acyl-CoA is a cosubstrate of the Eis enzyme tested.

^bData from ref 11. ^cData from ref 12.

NEO), four cases for Eis_Msm1 (HYG, KAN, NEO, and NET), and two combinations for Eis_Ava (NET and SIS) and Eis_Krh (HYG and RIB). These results were equal to or greater than the two cases where *n*-propionylation and acetylation were equal with Eis_Mtb (KAN and NEO).¹²

For those combinations that displayed a number of *n*-propionylations lower than that of acetylations, additional experiments were performed to establish if Eis homologues could further acetylate these *n*-propionylated AGs. Sequential acylation reactions in which the enzymes were first allowed to catalyze the *n*-propionylation of the AG for 24 h prior to addition of AcCoA were performed (Tables 4 and S3–S9 and Figures S6, S9, S13, S17, S21, S24, and S28). From these sequential experiments, one could expect to see that (1) no acetyl moiety can be transferred to an *n*-propionylated AG, indicating that *n*-propionylation prevents further acetylation; (2) the total number of modifications (acetylation and *n*-propionylation) equals that of acetylations when only AcCoA is used, indicating that the AG-binding site is likely sufficiently large to accommodate the *n*-propionylated AGs in the same orientation as their corresponding acetylated versions for additional acetylations; or (3) the total number of modifications is less than the number of acetylations observed in the presence of AcCoA alone, likely pointing to the fact that the AG-binding pocket is not large enough to accommodate the extra bulkiness of the *n*-propionyl group on modified AGs in specific orientations in the cavity. In most cases, additional acetylation of *n*-propionylated AGs did occur. The one exception was that of Eis_Ban, for which no additional acetylations were observed after *n*-propionylation with the two AGs tested, KAN and PAR. Other combinations where the *n*-propionylation stopped any further modifications included Eis_Ava with AMK, NEA, and PAR; Eis_Krh with SIS; and finally, Eis_Msm1 with NET, RIB, and SIS. Eis_Mtb showed limitation with propionylated NET, RIB, and SIS. Although additional acetylations are possible, these results demonstrate that the inability of AGs to become modified with ProCoA as efficiently as AcCoA may lie in the kinetics of the *n*-propionyl transfer rather than the bulkiness of the extra methyl group of the cosubstrate. The remaining results are spread between scenarios 2 and 3. These data indicate that the loss of modification is due primarily to the binding of the modified AG to the enzyme (scenario 3) or that *n*-propionylation has no effect on acetylation (scenario 2). All observed combinations are presented in Tables 4 and S3–S9.

Cosubstrate Competition of Eis Enzymes. Due to the abundance of both AcCoA and ProCoA in the cellular environment, a competition experiment was performed to gain insight into the kinetic mechanism of the enzymes (Tables 4 and S3–S9 and Figures S7, S10, S14, S18, S22, S25, and S29). AGs were incubated with Eis homologues and a 1:1 mixture of AcCoA and ProCoA and the results analyzed. From these competition experiments, one could expect to observe (1) no *n*-propionylation only acetylation, (2) no acetylation only *n*-propionylation, (3) mixed *n*-propionylation and acetylation, or (4) lower levels of acetylation than incubation with AcCoA alone, due to a competition with the ProCoA for the CoA-binding site. There were no observed cases that follow the second hypothesized observation, meaning that the presence of AcCoA limits the amount of *n*-propionylation that can occur. The most common observation was scenario 3, where there is a majority of acetylation with some *n*-propionylation (usually mono-), followed closely by scenario 4, where less acetylation is observed, likely due to reaction inhibition due to an abundance of CoA-containing molecules. From all these data arise a few interesting cases including NEO with Eis_Ban, by which under pure reaction, either AcCoA or ProCoA, only trimodification was observed. When AcCoA and ProCoA are combined, the maximum modification observed is still trimodification (mono-Ac-di-Pro-NEO); however, only diacetyl or dipropionyl-NEO is observed for NEO molecules modified by a single CoA species. Another interesting outcome was the reaction of Eis_Ban with PAR; in this reaction we saw monoacetyl-dipropionylated PAR. In the sequential reaction only dipropionylated PAR was observed. These data suggest that acetylation occurred either first or second and *n*-propionylation then occurred. Also of interest is the reaction of Eis_Gbr with NEO, after which a whole spectrum of modifications was observed. Everything from triacetyl-NEO to tripropionyl-NEO, including the two possible acetyl-propionyl mixed products, was observed as seen in Tables 4 and S3–S9.

Inhibition of Eis Homologues with Eis_Mtb Inhibitors. Knowing that the active sites of the Eis homologues are similar enough to acetylate a variety of AGs and CAP, but different enough to display diverse results, we wondered how these similarities and differences would be reflected in the inhibition of enzymatic activity. Chlorhexidine (Figure 1C), an inhibitor of Eis_Mtb,¹⁹ was tested against all seven AG-modifying Eis homologues (Table 5). Although chlorhexidine is used as a topical antibiotic, we used the compound here simply to test if

Table 4. Sequential Reactions Using ProCoA Followed by AcCoA and Competition Assays in Which Reactions Were Performed with both AcCoA and ProCoA^a

AG	ProCoA → AcCoA ^b							
	Eis_Ava	Eis_Ban	Eis_Gbr	Eis_Krh	Eis_Mab	Eis_Msm1	Eis_Mtb ^c	Eis_Tpa
AMK	mono-Ac mono-Pro	ND	ND	mono-Ac-mono-Pro	×	mono-Ac-mono-Pro di-Pro mono-Ac-di-Pro	mono-Ac-di-Pro	di-Pro mono-Ac-di-Pro
HYG	×	ND	ND	ND	ND	ND	mono-Pro	mono-Pro mono-Ac-mono-Pro
KAN	ND	mono-Pro	ND	di-Ac mono-Ac-mono-Pro	ND	ND	di-Pro	ND
NEA	di-Pro	ND	mono-Pro mono-Ac-mono-Pro di-Pro di-Ac-mono-Pro mono-Ac-di-Pro	mono-Ac-mono-Pro	ND	mono-Ac-Mono-Pro di-Pro di-Ac-mono-Pro mono-Ac-di-Pro	mono-Ac-di-Pro	ND
NEO	di-Ac mono-Ac-mono-Pro mono-Ac-di-Pro	ND	ND	mono-Ac-di-Pro	mono-Ac-di-Pro	ND	tri-Pro	di-Ac-Pro mono-Ac-di-Pro tri-Ac-Pro di-Ac-di-Pro
NET	ND	ND	ND	mono-Ac mono-Pro di-Ac mono-Ac-mono-Pro	ND	mono-Pro	mono-Pro	ND
PAR	mono-Ac-mono-Pro di-Pro	di-Pro	mono-Ac-di-Pro	mono-Ac-di-Pro	ND	mono-Ac-mono-Pro di-Pro di-Ac-mono-Pro mono-Ac-di-Pro	mono-Ac-mono-Pro	ND
RIB	mono-Pro di-Ac mono-Ac-mono-Pro di-Pro mono-Ac-di-Pro	ND	ND	ND	ND	di-Pro	di-Pro	ND
SIS	ND	ND	ND	mono-Pro mono-Ac-mono-Pro di-Pro	ND	mono-Pro	mono-Pro	ND
AG	AcCoA + ProCoA ^d							
	Eis_Ava	Eis_Ban	Eis_Gbr	Eis_Krh	Eis_Mab	Eis_Msm1	Eis_Mtb ^c	Eis_Tpa
AMK	mono-Ac di-Ac mono-Ac-mono-Pro	di-Ac	mono-Ac mono-Ac-mono-Pro	mono-Ac di-Ac mono-Ac-mono-Pro	×	di-Ac	di-Ac tri-Ac	di-Ac
HYG	×	mono-Ac di-Ac	mono-Ac	mono-Ac	mono-Ac	mono-Ac	mono-Ac mono-Ac-mono-Pro	mono-Ac di-Ac

Table 4. continued

AG	AcCoA + ProCoA ^d							
	Eis_Ava	Eis_Ban	Eis_Gbr	Eis_Krh	Eis_Mab	Eis_Msm1	Eis_Mtb ^e	Eis_Tpa
KAN	mono-Ac	mono-Ac	mono-Ac mono-Pro	di-Ac	di-Ac	di-Ac	di-Ac	di-Ac
NEA	mono-Ac mono-Pro di-Ac tri-Ac	di-Ac tri-Ac di-Ac-mono-Pro mono-Ac-di-Pro	mono-Ac mono-Pro	di-Ac	tri-Ac di-Ac-mono-Pro	di-Ac	tri-Ac di-Ac-mono-Pro	di-Ac tri-Ac
NEO	mono-Ac di-Ac mono-Ac-mono-Pro	di-Ac mono-Ac-mono-Pro di-Pro mono-Ac-di-Pro	di-Ac di-Pro tri-Ac di-Ac-mono-Pro mono-Ac-di-Pro tri-Pro	di-Ac mono-Ac-mono-Pro	tri-Ac di-Ac-mono-Pro tetra-Ac tri-Ac-mono-Pro di-Ac-di-Pro	tri-Ac tri-Pro	tri-Ac tri-Pro	mono-Ac mono-Pro di-Ac mono-Ac-mono-Pro tri-Ac
NET	mono-Ac mono-Pro mono-Ac-mono-Pro	mono-Ac mono-Pro	mono-Ac mono-Pro	mono-Ac	mono-Ac di-Ac mono-Ac-mono-Pro di-Pro	mono-Ac	mono-Ac di-Ac	mono-Ac di-Ac mono-Ac-mono-Pro
PAR	di-Ac tri-Ac	di-Ac mono-Ac-di-Pro	di-Ac mono-Ac-mono-Pro	di-Ac	tri-Ac	di-Ac	di-Ac	di-Ac tri-Ac
RIB	di-Ac mono-Ac-mono-Pro	mono-Ac mono-Pro di-Ac	mono-Ac mono-Pro di-Ac	di-Ac	di-Ac	mono-Ac di-Ac	di-Ac	di-Ac
SIS	mono-Ac di-Ac mono-Ac-mono-Pro	mono-Ac di-Ac mono-Ac-mono-Pro	mono-Ac di-Ac	mono-Ac di-Ac mono-Ac-mono-Pro	di-Ac	mono-Pro	mono-Ac di-Ac mono-Ac-mono-Pro	di-Ac

^aX indicates that the reaction was not tested as the AG was found to not be a substrate of the enzyme; ND indicates that the reaction was not tested because the number of *n*-propionylations was equal to the number of acetylations. ^bReactions with ProCoA were followed by incubation with AcCoA. ^cData from ref 12. ^dAcCoA and ProCoA were incubated with Eis and AG in a 1:1 ratio.

Table 5. IC₅₀ Values of the Known Eis_Mtb Inhibitor Chlorhexidine with Eis Homologues

Eis species	chlorhexidine (μM)	IC ₅₀ Xxx/IC ₅₀ Mtb
Ava	20 ± 7 ^a	111
Ban	14 ± 4 ^b	77
Gbr	4.4 ± 0.6	23
Krh	17 ± 4	91
Mab	4.4 ± 0.9	23
Msm1	1.9 ± 0.4 ^a	10
Mtb	0.188 ± 0.030 ^c	1
Tpa	7.4 ± 1.6	40

^aData from ref 11. ^bData from ref 37. ^cData from ref 19.

the inhibitor would broadly inhibit all Eis homologues or be specific to Eis_Mtb or Eis from mycobacteria. Chlorhexidine displayed a wide range of activities for the Eis homologues, ranging from low nanomolar (Eis_Mtb) to micromolar (Eis_Ava and Eis_Ban). This is an approximate 100-fold range in IC₅₀ value, indicating that this inhibitor targets a broad range of Eis homologues. Generally the trend seems to follow that the less homologous the protein is to Eis_Mtb, the higher

the IC₅₀ value, implying a less potent inhibitor. Whereas chlorhexidine would be a poor candidate as an antitubercular drug, it does tell us that inhibitors of Eis_Mtb may inhibit other homologues, but an increase in the IC₅₀ value could be expected.

In summary, the consolidation of mass spectral analysis, kinetic, inhibitory, and activity data tells us that the Eis homologues share activity, cosubstrate tolerance, and modification multiplicity. We have shown that deletion of 13 residues can render an Eis homologue inactive, and an additional 10 kDa can have a limited effect on activity. Although without structural data these hypotheses are speculative, we have learned that Eis homologues can be found in a diverse community of bacteria and that they behave similarly to Eis_Mtb. They also have very distinct sequences, kinetic profiles, and inhibition. Whereas the enzymes do not all come from pathogenic bacteria, the similarity of the enzymes encoded in the genomes of these nonpathogenic species may be a warning that although currently the species do not pose a threat, they may become more aggressive in the future.

METHODS

Phylogenetic Tree Generation. The amino acid sequences of 22 Eis homologues were obtained from NCBI through the Geneious Pro 4.8.5 program to construct a phylogenetic tree (Figure 2). The parameters used were as follows: Jukes–Cantor genetic distance and cost matrix Blosum45. The Eis homologue accession numbers from NCBI were YP_325469 (*Ava*), YP_029001 (*Ban*), YP_003272441 (*Gbr*), YP_001855041 (*Krh*), YP_00170485 (*Mab1*), YP_001705255 (*Mab2*), ZP_05216001 (*Mav1*), ZP_05217647 (*Mav2*), ZP_05214704 (*Mav3*), EKF23162 (*Mha*), ZP_05228133 (*Min1*), ZP_05227568 (*Min2*), YP_005335697 (*Min3*), ZP_04749484 (*Mka1*), ZP_04747464 (*Mka2*), ZP_06852004 (*Mpa1*), ZP_06848356 (*Mpa2*), ZP_06849426 (*Mpa3*), YP_887817 (*Msm1*), YP_888812 (*Msm2*), AAF03768.1 (*Mtb*), and YP_003645809 (*Tpa*). The percent identity of these homologues to Eis_ *Mtb* is presented in Table S1.

Materials and Instrumentation. Chemically competent *Escherichia coli* (TOP10 and BL21 (DE3)) cells were purchased from Invitrogen (Carlsbad, CA, USA). Genomic DNA (*Krh* and *Tpa*) and bacterial strains (*Gbr*, *Mab*, *Mav*, *Min*, *Mka*, and *Mpa*) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Genomic DNA for *Mha*³⁵ was a gift from Dr. Nuno Empadinhas (University of Coimbra, Portugal). The pET28a plasmid used in this study was obtained from Novagen (Gibbstown, NJ, USA). All primers used were obtained from Integrated DNA Technologies (Coraville, IA, USA). All cloning enzymes including restriction enzymes, Phusion DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Sequencing of generated plasmids was performed at the University of Michigan DNA sequencing core. AMK, CAM, CAP, CIP, KAN, NEO, NOR, RIB, SIS, SPT, STR, AcCoA, acyl-CoAs (ProCoA, *n*-butyryl-CoA, *n*-hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, crotonyl-CoA, malonyl-CoA, and *i*-valeryl-CoA), and dithionitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used without further purification. NEA, NET, and PAR were purchased from AK Scientific (Mountain View, CA, USA). All UV–vis assays and kinetic experiments were performed using a multimode SpectraMax M5 plate reader in 96-well plates from Fisher Scientific (Pittsburgh, PA, USA). Liquid chromatography–mass spectrometry (LC-MS) chromatograms were obtained on a Shimadzu LC-MS-2019EV equipped with an SPD-20AV UV–vis detector and an LC-20AD liquid chromatograph. The PDB structures 2OZG (Eis_ *Ava*),¹¹ 3N7Z (Eis_ *Ban*),³⁷ 3SXN (Eis_ *Msm1*),¹⁷ and 3R1K (Eis_ *Mtb*)² were visualized using PyMOL (The PyMOL Molecular Graphics System, version 1.4.1, Schrödinger, LLC).

DNA Isolation from Bacteria. DNA was isolated from the following bacteria: *M. abscessus* ATCC 19977, *M. kansasii* ATCC 12478, *M. parascrofulaceum* ATCC BAA-614, *M. intracellulare* ATCC 13950, *M. avium* ATCC 25291, and *G. bronchialis* ATCC 25592. Mycobacteria were grown in 7H9 medium with albumin–dextrose–catalase (ADC). One milliliter of turbid culture was pelleted (13000 rpm, 1 min, room temperature) and resuspended in TE buffer (400 μ L). The bacteria were inactivated with heat (30 min, 80 $^{\circ}$ C). After cooling to room temperature, to avoid clumping of the cells, four to five glass beads were added and the cells were briefly

vortexed. Lysozyme (30 μ L, 50 mg/mL) was added, and the suspension was further incubated for 1 h at 37 $^{\circ}$ C. At this point SDS (10 μ L, 10%) and proteinase K (10 μ L, 20 mg/mL) were added, and the suspension was gently vortexed, followed by a brief incubation period (15 min, 65 $^{\circ}$ C). NaCl (100 μ L, 5 M) and CTAB/NaCl (100 μ L, 10% CTAB in 0.7 M NaCl) were added sequentially, and the suspension was further incubated (10 min, 65 $^{\circ}$ C). The mixture was extracted with phenol/chloroform/isoamyl alcohol 25:24:1 (3 \times 750 μ L). The DNA was precipitated using isopropanol (0.6 \times volume) and incubated (20 min, –20 $^{\circ}$ C). The DNA was collected by centrifugation (12000 rpm, 10 min, room temperature), the isopropanol was removed, and the DNA pellet was washed with ice-cold ethanol (75%, 1 mL) and centrifuged (12000 rpm, 5 min, room temperature). The ethanol was removed and allowed to air-dry (10 min, room temperature). The DNA was redissolved in TE buffer (100 μ L, 30 min, 37 $^{\circ}$ C). *G. bronchialis* was grown in BHI, and DNA was isolated as above.

Cloning, Expression, and Purification of Eis Proteins. Eis_ *Mtb*,² Eis_ *Msm1*,¹⁰ Eis_ *Ban*,³⁷ and Eis_ *Ava*¹¹ were cloned and purified as previously described. The remaining Eis proteins were cloned using the isolated DNA, the corresponding primers reported in Table S2, and Phusion DNA polymerase. The isolated PCR fragments were inserted into a linearized pET28a vector between the *NdeI* (all genes) and *XhoI* (Eis_ *Gbr*, Eis_ *Krh*, Eis_ *Mab1*, Eis_ *Mab2*, Eis_ *Mha*, and Eis_ *Tpa*) or *HindIII* (Eis_ *Mav1*, Eis_ *Mav2*, Eis_ *Mav3*, Eis_ *Min1*, Eis_ *Min2*–3, Eis_ *Mka*, Eis_ *Mpa*, and Eis_ *Msm2*) restriction sites. The plasmids containing the *eis* genes were then transformed into *E. coli* TOP10 chemically competent cells. Sequencing of the isolated plasmid revealed perfect alignment with the reported gene sequences (Table S1). Plasmids were transformed into *E. coli* BL21 (DE3) chemically competent cells for protein expression. All new Eis enzymes were purified exactly as indicated for Eis_ *Mtb*.² All proteins were dialyzed in Tris-HCl buffer (50 mM, pH 8.0 adjusted at room temperature) and stored at 4 $^{\circ}$ C. After purification, 2.2 mg (Eis_ *Gbr*), 1.8 mg (Eis_ *Krh*), 0.1 mg (Eis_ *Mab1*), 0.4 mg (Eis_ *Mab2*), 3.8 mg (Eis_ *Mav1*), 0.8 mg (Eis_ *Mav2*), 0.5 mg (Eis_ *Mav3*), 7.0 mg (Eis_ *Mha*), 2.6 mg (Eis_ *Min1*), 1.2 mg (Eis_ *Min2/3*), 1.0 mg (Eis_ *Mka1*), 0 mg (Eis_ *Mka2*, insoluble), 0.4 mg (Eis_ *Mpa1*), 0.5 mg (Eis_ *Mpa2*), 0.3 mg (Eis_ *Mpa3*), 0.5 mg (Eis_ *Msm2*), and 1.9 mg (Eis_ *Tpa*) of protein were obtained per liter of culture. SDS-PAGE gels showing all Eis homologue proteins purified are presented in Figure S3.

Spectrophotometric Measurements of Acyltransferase Activity. The acyltransferase activity of Eis enzymes was tested against a variety of antibiotics using AcCoA, by monitoring the 2-nitro-5-thiobenzoate (NTB[–]) produced by the reaction of free CoA with DTNB. Reactions were monitored at 412 nm ($\epsilon_{412\text{ nm}} = 14150\text{ M}^{-1}\text{ cm}^{-1}$) for 1 h, taking measurements every 30 s at 25 $^{\circ}$ C. Reaction mixtures (200 μ L) containing antibiotic (100 μ M), AcCoA (500 μ M), DTNB (2 mM), and Tris-HCl buffer (50 mM, pH 8.0 adjusted at room temperature) were initiated by the addition of Eis enzyme (0.5 μ M). The only non-AG to be modified by Eis enzymes was CAP (Figure 1B); UV–vis curves for this antibiotic are presented in Figure 5. All enzymes displaying activity against AGs were also tested for cosubstrate promiscuity (Table 3). Here acyl-CoAs (ProCoA, *n*-butyryl-CoA, *n*-hexanoyl-CoA, octanoyl-CoA, decaoyl-CoA, lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, crotonyl-CoA, malonyl-

CoA, and *i*-valeryl-CoA) (500 μ M) were tested against NEO (100 μ M), the most dynamic AG with all AG-active enzymes.

Determination of Number of Acetylations and *n*-Propionylations as well as Sequential and Competitive Modifications of AGs by Eis Proteins via LC-MS. The extent of acetylation for each AG that was found to be active by UV-vis assays (Figure 1A) was determined by using LC-MS. Reactions (30 μ L) containing AG (AMK, HYG, KAN, NEA, NEO, NET, PAR, RIB, and SIS, 0.67 mM), AcCoA (3.35 mM), Eis enzyme (5 μ M), and Tris-HCl buffer (50 mM, pH 8.0 adjusted at room temperature) were incubated overnight at room temperature. Reactions were quenched by the addition of ice-cold methanol (30 μ L) and were chilled (-20 $^{\circ}$ C) for at least 20 min. To remove excess enzyme from the solution, the reactions were then centrifuged (13000 rpm, 10 min, room temperature) and diluted 1:1 with H₂O before loading onto the LC-MS. Samples were run using H₂O (0.1% formic acid). A summary of the level of acetylation is presented in Table 1. The *n*-propionylation of AGs (AMK, HYG, KAN, NEA, NEO, NET, PAR, RIB, and SIS) was also monitored using the above method substituting ProCoA for AcCoA. A summary of *n*-propionylation is also presented in Table 1. Sequential acylations (*n*-propionylation followed by acetylation) were performed and monitored as above. After the first overnight incubation with ProCoA, AcCoA (3.35 mM) and additional Eis enzyme (5 μ M) were added, bringing the total volume up to 40 μ L, and incubated for an additional 24 h. A summary of the acylated AG species obtained during sequential experiments is presented in Table 4. Competition experiments were also performed as above in the simultaneous presence of both AcCoA (3.35 mM) and ProCoA (3.35 mM). A summary of the acylated AG species obtained during competition experiments is also presented in Table 4. Calculated and observed masses for all AGs against all Eis homologue proteins are presented in Tables S3–S9, with each table summarizing findings for a specific Eis homologue. Mass spectra for all of these experiments are presented in Figures S5–S29 for Eis_Ava, Eis_Ban, Eis_Gbr, Eis_Krh, Eis_Mab2, Eis_Msm1, and Eis_Tpa in the following order: (i) acetylation, (ii) *n*-propionylation, (iii) sequential *n*-propionylation followed by acetylation, and (iv) competition between *n*-propionylation and acetylation.

Determination of Steady-State Kinetic Parameters of Eis Proteins for AGs. The kinetic parameters (k_{cat} and K_m) were determined for acetylation with KAN and PAR for all enzymes, in reactions (200 μ L) with a fixed AcCoA (0.5 μ M) concentration. For Eis_Krh, Eis_Mab2, and Eis_Tpa, concentrations of 0, 20, 50, 100, 250, 500, 1000, and 2000 μ M were used with both AGs. With Eis_Gbr the ranges varied, KAN concentrations were 0, 20, 50, 250, 500, and 1000 μ M, and PAR concentrations were 0, 20, 50, 100, 250, 500, 1000, and 2000 μ M. The reaction cocktail containing DTNB (2 mM), Tris-HCl buffer (50 mM, pH 8.0 adjusted at room temperature), AcCoA (0.5 μ M), and Eis enzyme (0.25 μ M) was initiated by the addition of the AGs. The reactions were monitored at 412 nm by taking readings every 20 s for 20 min at 25 $^{\circ}$ C. The determination of kinetic parameters was done with a nonlinear regression using Sigma-Plot 11.0 software (Systat Software Inc., San Jose, CA, USA) (Table 2 and Figure S4).

Inhibition of Eis Enzymes. IC₅₀ values for chlorhexidine (Figure 1C) were determined as previously reported.^{10,11,19} Briefly, reactions (200 μ L) were initiated in a stepwise manner.

Chlorhexidine (100 μ L) was dissolved in Tris-HCl buffer (50 mM, pH 8.0, 10% DMSO) and subjected to a 5-fold serial dilution. To the chlorhexidine solution was added a mixture (50 μ L) of Tris-HCl buffer (50 mM, pH 8.0), Eis enzyme (2 μ M), and NEO (400 μ M) followed by incubation (10 min, room temperature). A third reaction mixture consisting of Tris-HCl buffer (50 μ M, pH 8.0), AcCoA (2 mM), and DTNB (8 mM) was used to initiate the reactions. The reactions were monitored as described for the kinetic analysis. The determination of IC₅₀ values was done with a Hill plot analysis using Kaleidagraph 4.1 software, and the data are summarized in Table 5.

■ ASSOCIATED CONTENT

📄 Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00036.

9 tables displaying nucleotide and protein sequence numbers for the various Eis homologues studied (Table S1), primers used for cloning (Table S2), and mass analysis for AGs acylated by Eis homologues (Tables S3–S9); 29 figures presenting alignments of Eis homologue protein sequences (Figures S1 and S2), SDS-PAGE gel of purified Eis homologues (Figure S3), representative Michaelis–Menten kinetic plots (Figure S4), and mass spectra of AGs acylated by Eis homologues (Figures S5–S29) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*(S.G.-T.) E-mail: sylviegttsodikova@uky.edu. Phone: (859) 218-1686. Fax: (859) 257-7585.

Notes

The authors declare no competing financial interest.

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