Bacterial fatty acid synthesis in *Escherichia coli* is initiated by the condensation of an acetyl-CoA with a malonyl-acyl carrier protein (ACP) by the β-ketoacyl-ACP synthase III enzyme, FabH. *E. coli* ΔfabH knockout strains are viable because of the *yiiD* gene that allows FabH-independent fatty acid synthesis initiation. However, the molecular function of the *yiiD* gene product is not known. Here, we show the *yiiD* gene product is a malonyl-ACP decarboxylase (MadA). MadA has two independently folded domains: an amino-terminal N-acetyl transferase (GNAT) domain (MadAN) and a carboxy-terminal hot dog dimerization domain (MadAC) that encodes the malonyl-ACP decarboxylase function. Members of the proteobacterial Mad protein family are either two domain MadA (GNAT-hot dog) or standalone MadB (hot dog) decarboxylases. Using structure-guided, site-directed mutagenesis of MadB from *Shewanella oneidensis*, we identified Asn45 as a key catalytic residue. MadA, MadAC, or MadB expression all restored normal cell size and growth rates to an *E. coli* ΔfabH strain, whereas the expression of MadAN did not. Finally, we verified that GlmU, a bifunctional glucosamine-1-phosphate N-acetyl transferase/N-acetyl-glucosamine-1-phosphate uridylyltransferase that synthesizes the key intermediate UDP-GlcNAc, is an ACP binding protein. Acetyl-ACP is the preferred glucosamine-1-phosphate N-acetyl transferase/N-acetyl-glucosamine-1-phosphate uridylyltransferase substrate, in addition to being the substrate for the elongation-condensing enzymes FabB and FabF. Thus, we conclude that the Mad family of malonyl-ACP decarboxylases supplies acetyl-ACP to support the initiation of fatty acid, lipopolysaccharide, peptidoglycan, and enterobacterial common antigen biosynthesis in Proteobacteria.

Bacterial type II fatty acid synthesis (FASII) is catalyzed by a collection of conserved enzymes that supply fatty acids for membrane phospholipid synthesis and has been the subject of intense study for decades (1). Initially, FASII was thought to be initiated by an acetyl-CoA:acyl carrier protein (ACP) transacylase to generate an acetyl-ACP primer and an enzyme that catalyzes this reaction was purified (2). In this original hypothesis, the acetyl-ACP is then used by the elongation-condensing enzymes (FabB or FabF) to initiate cycles of elongation (2–4). In 1987, an enzyme was discovered that catalyzes the condensation of acetyl-CoA with malonyl-ACP in *Escherichia coli* to initiate FASII (5), and the fabH gene encoding this activity was identified in 1992 (6). FabH homologs are widely distributed and are the major enzymes responsible for the initiation of FASII (1) and are targets for antibiotic drug discovery (7, 8). FabH is a ping-pong enzyme and also catalyzes acetyl-CoA:ACP transacylation albeit at a much lower rate than the condensation reaction (9), suggesting that FabH may be responsible for the transacylation activity noted previously (2). In *E. coli*, fabH was thought to be an indispensable gene because knockout strains could not be derived (10). These experiments were performed in a strain with two mutations (*relA1* and *spoT1*) that perturb the regulation of the alarmone ppGpp. However, ΔfabH and ΔspoT are synthetically lethal, and ΔfabH strains are recovered in either a WT or *relA1* background (11). Although these ΔfabH strains grew significantly slower and had a smaller cell size than the WT, this result meant that there was another enzyme that could partially substitute for FabH. Sanyal et al. (12) identified this gene as *yiiD*, but the catalytic activity of YiiD remains unknown.

This study identifies YiiD as a malonyl-ACP decarboxylase (MadA) that produces acetyl-ACP. MadA has an amino terminal N-acetyl transferase (GNAT) domain (MadAN) fused to a carboxy terminal hot dog-fold domain (MadAC) encoding the malonyl-ACP decarboxylase function. MadB is a standalone hot dog malonyl-ACP decarboxylase from *Shewanella oneidensis*, and structure-guided mutagenesis identifies Asn45 as a key catalytic residue. MadA, MadAC, or MadB expression restores cell size and growth rate to an *E. coli* ΔfabH strain, whereas the expression of MadAN does not. We verify that GlmU (glucosamine-1-phosphate N-acetyl transferase/N-acetyl-glucosamine-1-phosphate uridylyltransferase) is an ACP binding protein and show that acetyl-ACP is the preferred GlmU substrate for the synthesis of UDP-GlcNAc. Thus, the Mad family of malonyl-ACP decarboxylases supplies acetyl-ACP to support the initiation of FASII, lipopolysaccharide (LPS), peptidoglycan (PG) and
enterobacterial common antigen (ECA) biosynthesis in Proteobacteria.

**Results**

**MadA (YiiD) is a malonyl-ACP decarboxylase**

We first tested whether the purified MadA (Fig. S1A) catalyzed a condensing enzyme reaction that replaced FabH function using a coupled enzyme assay according to the scheme outlined in Figure 1A. FabD was used to produce malonyl-ACP that was converted to 3-ketobutyryl-ACP by FabH in the presence of acetyl-CoA. The 3-ketobutyryl-ACP product is not an abundant product, so FabG was used in a coupled reaction to reduce 3-ketobutyryl-ACP to the stable 3-hydroxybutyryl-ACP (13–15). The samples were separated on urea gels that resolve the different ACP thioesters based on their ability to stabilize ACP conformation (13–15). FabH formed 3-ketobutyryl-ACP and 3-hydroxybutyryl-ACP in the coupled enzyme system from [14C]acetyl-CoA, but MadA did not (Fig. 1A), showing that MadA does not carry out a FabH-like condensing enzyme reaction. In addition, MadA is not an acetyl-CoA:ACP acetyltransferase because [14C]acetyl-ACP was not detected in these experiments. When [2-14C]malonyl-CoA was used as substrate, [14C]malonyl-ACP was formed and converted by FabH to 3-hydroxybutyryl-ACP (Fig. 1A). However, [2-14C]malonyl-ACP was converted to a different product by MadA that appeared to be [14C]acetyl-ACP based on its electrophoretic mobility (Fig. 1A).

Assays containing only MadA and [2-14C]malonyl-ACP verified that MadA was necessary and sufficient for acetyl-ACP formation (Fig. 1B). The [2-14C]malonyl-ACP and [14C]acetyl-ACP standards were synthesized using AcpS and apo-ACP to create the labeled acyl-ACPs from their respective labeled acyl-CoAs. Commercial [2-14C]malonyl-CoA is contaminated with a few percent [14C]acetyl-CoA, and AcpS prefers acetyl-CoA over malonyl-CoA (16) resulting in the unavoidable contamination of the [2-14C]malonyl-ACP substrate preparation with a detectable level of [14C]acetyl-ACP product (Fig. 1B). MadA addition results in the dose-dependent decarboxylation of [2-14C]malonyl-ACP to [14C]acetyl-ACP (Fig. 1B). The MadA product was verified as acetyl-ACP by mass spectrometry (Fig. 1C). The reactions were performed with nonradioactive substrates, the samples were acid precipitated, and the pellets hydrolyzed with Asp-N protease. This process liberates a tripeptide harboring the prosthetic group and attached acyl chains that were then separated and detected by LC-MS/MS (17–19). Acetyl-ACP was not detected in assays without MadA and was robustly detected in assays containing MadA (Fig. 1C). Acetyl-ACP is readily used by *E. coli* FabB or *Streptococcus pneumoniae* FabF-condensing enzymes (Fig. 1D), confirming that acetyl-ACP is used to initiate FASII by these enzymes. These data verify acetyl-ACP as the MadA product.
product and identify the product of the yiiD gene as a Mad. 
Sanyal et al. called yiiD fabY (12); however, the FabY name was designated as a bona fide FabH-condensing enzyme homolog from Pseudomonas aeruginosa in 2012 (20). Now that the function of YiiD is known, we designate the yiiD gene as madA.

Analytical ultracentrifugation verified that MadA is a dimeric ACP-binding protein (Table S1). ACP is a monomer (Fig. 2A), and MadA was confirmed as a dimer by sedimentation equilibrium analysis (Fig. 2B). The analysis of MadA plus ACP resulted in the appearance of a new 92-kDa protein species that corresponds in molecular weight to a MadA-(ACP)₂ complex (Fig. 2C) meaning that there are two ACP-binding sites per MadA dimer.

MadA has two independent domains

The bioinformatic analysis of MadA indicates that it is composed of two distinct protein domains, MadAN and MadAC (Fig. 3A). The amino terminal MadAN domain has distinct homology to protein acetyltransferases in the GNAT superfamily. These proteins typically use acetyl-CoA to acylate primary amines such as lysine amino groups on proteins (21). Specifically, MadAN belongs to the Acetyltransf_1 protein family (Pfam_00583) and is most closely related to the PDB entry for a GNAT acetyltransferase of unknown function from Staphylococcus aureus (PDB ID: 5JQ4). Mapping the MadAN sequence onto this acetyltransferase structure suggests that the acetyltransferase domain of MadAN lies between residues 19 and 162 (Fig. 3A). The carboxy terminal domain has a predicted hot dog fold associated with enzymes that carry out thioesterase, hydratase, or dehydratase reactions (22–24). The MadAC domain belongs to Pfam_09500, which consists of highly related hot dog proteins that are widely expressed in Proteobacteria and are annotated as thioesterases. Mapping the MadAC sequence onto the structure of a Pfam_09500 member from S. oneidensis (PDB ID: 1T82) suggests that the MadAC domain encompasses residues 171 to 314 (Fig. 3A). These two domains are connected by a 10 amino acid region of low complexity containing three proline and five threonine residues, indicating that MadA consists of two independently folded domains connected by a flexible spacer. We constructed MadAN and MadAC recombinant proteins as outlined in Figure 3A. MadAN migrated as a monomer (Fig. S1B) and the MadAC migrated as a dimer (Fig. S1C) by gel filtration chromatography. Sedimentation equilibrium analysis confirmed that MadAN is a monomer (Fig. S2A) and MadAC is a dimer (Fig. S2B) (Table S1). Thus, MadAC encodes the MadA dimerization domain.

The thermal denaturation profiles of the recombinant proteins showed MadA has two distinct structural transitions at 38 °C and 69 °C (Fig. 3B). This unique denaturation pattern supports the idea that MadA consists of two independently folded and noninteracting protein domains. Purified MadAN was a compactly folded protein with a denaturation temperature of 47 °C and MadAC denatured at 75 °C (Fig. 3B). The isolated individual domains are more stable than when they are tethered together in MadA. The individual domains were tested for malonyl-ACP decarboxylase activity. MadAN did not catalyze the malonyl-ACP decarboxylase reaction, whereas MadAC, like MadA, is an active malonyl-ACP decarboxylase (Fig. 3C). Thus, MadAC is an independently folded hot dog dimerization domain that is necessary and sufficient for malonyl-ACP decarboxylase activity.

**MalB malonyl-ACP decarboxylases**

There are 740 sequences containing the MadAC domain architecture in Pfam_09500. Among those, 675 occur in Proteobacteria, 20 are in Verrucomicrobia, 11 are found in
Firmicutes, and the remaining 34 are scattered among 12 additional phyla (Table S2). The most common protein architecture is a standalone MadA domain occurring in 470 of the 740 sequences. We call the standalone members of Pfam_09500 MadB. There are 270 of the 740 sequences with the MadA domain fused to an amino terminal domain belonging to a member of the GNAT family of acyltransferases. The E. coli MadA is a fusion of the hot dog domain to an Acetyltrans_1 GNAT domain (Pfam_00583) and is the most common two-domain organization occurring in 214 of the 270 sequences. The MadA proteins occur almost exclusively in the Gammaproteobacteria.

The prediction that MadB proteins in Pfam_09500 are malonyl-ACP decarboxylases was validated by characterizing the MadB of S. oneidensis, the only Mad protein with a high-resolution X-ray structure (PDB ID: 1T82) (25). The 18-kDa recombinant MadB purified as a dimer by gel filtration chromatography (Fig. S1D), and the dimeric state of MadB was confirmed by sedimentation equilibrium analysis (Fig. S2C). MadB was a stable protein with a denaturation temperature of 71 ± 0.2 °C. MadB possessed comparable malonyl-ACP decarboxylase activity with that of MadA (Fig. 3C). Thus, MadB of S. oneidensis, and by inference all MadA and MadB members of Pfam_09500, are malonyl-ACP decarboxylases.

The gel assay for Mad activity is time consuming and a required reagent, [2-14C]malonyl-ACP, is difficult to synthesize and spontaneously degrades to [14C]acetyl-ACP over time (Fig. S3A). Usually, acyl-ACP is stored at pH 6 to 7 to minimize hydrolysis of the base-labile thioester bond. However, the conversion of malonyl-ACP to acetyl-ACP is an additional stability problem that is accelerated by low pH, heat, and time (Fig. S3A). This stability is a characteristic of malonyl-ACP because malonyl-CoA does not degrade to acetyl-CoA under the same conditions (Fig. S3B). The activities of ACP-dependent enzymes are often assayed using acyl-CoAs as surrogate, low-affinity substrates (26). Therefore, we developed a Mad assay using the substrate analog malonyl-CoA as a reliably quantitative approach to measuring Mad activity (Fig. 3D). Malonyl-CoA is a substrate for the Mad proteins with a Km >5 mM and a correspondingly lower enzyme specific activity than with malonyl-ACP. These data confirm that malonyl-ACP is the high-affinity Mad substrate. However, the malonyl-CoA decarboxylase assay uses readily available, stable materials to assess Mad activity. MadA, MadA, and MadB are malonyl-phosphopantetheine thioester decarboxylases, but MadA is not (Fig. 3D).

Structure and mechanism of Mad

Each protomer of S. oneidensis MadB has a prototypical hot dog-fold consisting of a central, hydrophobic, 6-turn α-helix (hot dog) surrounded by a six-stranded antiparallel β-sheet to create a dimer with two anti-parallel α-helices wrapped by 12 β-strands (Fig. 4A). The dimer interface is formed by the interaction between the α-helices and the β3 strand from each protomer. A prominent feature in the structure is the loop between residues 44 and 50 (Fig. 4A), and bioinformatic analyses of Pfam_09500 sequences identifies this short segment as the most highly conserved patch of amino acids in the protein family (Fig. 4B). Asn45 and Phe51 are completely conserved in the Mad family, with Asn43 present in 97% of sequences. Phe51 has an obvious structural role in stabilizing the base of the loop by packing the phenyl ring into an adjacent hydrophobic pocket. Each of the loop residue side chains engage in interactions that stabilize the loop conformation, and in the case of Asn43, make direct contact with Asn45 (Fig. 4C). Site-directed mutagenesis of the MadB catalytic loop residues produced properly folded proteins based on their thermal stabilities and dimeric structures (Table S3), and each was analyzed for Mad activity using the malonyl-CoA decarboxylase assay (Table S4).
decarboxylase assay (Fig. 4D). The MadB(S54A) and MadB(D79A) mutants had nearly normal malonyl-CoA decarboxylase activity. The decarboxylation activity decreased by 10-fold in the MadB(N43A), MadB(H47A), and MadB(T49A) mutants, illustrating that mutations in any of these conserved-loop residues compromise activity. The key catalytic residue was identified as Asn45 based on the reduction of decarboxylase activity by >100-fold in MadB(N45A) (Fig. 4D). These experiments identify the Ile44-Met50 loop as the Mad active site. The methylmalonyl-ACP decarboxylase encoded within the limonene polyketide gene cluster (LmnK) has a double hot dog fold and also uses an asparagine residue on a similar loop to catalyze decarboxylation (27, 28).

The architecture of the Mad proteins in solution was analyzed by size-exclusion chromatography-small angle X-ray scattering (SEC-SAXS) (29–31). MadB has a homogenous particle distribution, and the normalized Kratky plot is a close fit to a compact globular protein (Fig. 5A). Accordingly, the MadB crystal structure fits into the ab initio electron density map calculated from the SAXS data (32) (Fig. 5A, inset). MadA was also a homogenous particle population of dimers with a particle dimension of Dmax = 139 Å and radius of gyration of Rg = 38.21 ± 0.11 Å (Table S4). The normalized dimensionless Kratky plot shows a primary peak with a shoulder at low q that does not converge to the q axis (Fig. 5B). The bell-shaped profile with a peak occurring at qRg > 1/3 is consistent with a protein with ordered domains tethered together by a linker rather than being arranged in a globular conformation like MadB (Fig. 5A). Three theoretical models (globular, alternating, or linear) for the domain organization of MadA in solution were generated (33), and the linear model best fit the SAXS data set (Fig. 5B). Both the alternating and globular models show a prototypical peak at qRg = 1/3 (~1.73) with a peak height of 3/e (~1/1) (34) but were a very poor fit to the data (Fig. 5B). The differences between the linear model and the data at mid q suggest MadA samples a spectrum of compact and extended conformations (Fig. 5B). The ab initio electron density maps generated from the MadA solution scattering data (32) were superimposed on the globular, alternating, and linear MadA models to visualize the quality of the fits (Fig. 5C). The unmodeled electron density in the linear fit is attributed to the hydration shell and the independent movement of the MadAΔN and MadAΔC domains. We interpret these experiments to mean that the MadAΔN domains are globular balls on chains connected to the hot dog MadAΔC dimer.

**Complementation of ΔfabH strains**

*E. coli* strain NR1769 (ΔfabH) lacks the major initiation enzyme and exhibits small cell and slow growth phenotypes (11, 12). A series of plasmids expressing different Mad proteins in the pBAD vector were used to assess the complementation of these phenotypes (Fig. 6). Immunoblotting showed that the His-tagged constructs were all expressed to approximately the same level (Fig. S4). The small cell phenotype was clearly visible in strain NR1769 (ΔfabH)/pBAD and was restored to normal in strain NR1769 (ΔfabH)/pMadA (Fig. 6A). This effect was quantified by selecting those cells within each field that possess an in-plane “figure 8” morphology for measurement (11) (Fig. 6A, red ovals). Cohorts of 50 cells were measured for each of the constructs and their lengths and widths plotted (Fig. 6B). The expression vectors harboring MadA, MadAΔC, and MadB were as effective as FabH in restoring cell size (both length and width) to the WT range. MadAΔN expression did not restore cell size. In addition, the plasmids expressing either
MadA, MadAC, or MadB were as effective as the FabH plasmid in correcting the doubling time defect in strain NR1769 (ΔfabH), but MadAN expression did not (Fig. 6C). The amount of MadA protein in the cell is about an order of magnitude lower than FabH (35) suggesting why the endogenous expression level of madA is not sufficient to complement the ΔfabH phenotypes. Thus, elevated malonyl-ACP decarboxylase activity completely restores the growth rate and cell size defects caused by the absence of FabH.

Acyl-CoA and acyl-ACP pools

The impact of FabH deletion and Mad complementation on the composition of the intracellular CoA and ACP thioester pools was examined in vivo. Acetyl-CoA is the major CoA thioester in WT cells, and there was an accumulation of malonyl-CoA coupled with a depletion of the nonesterified CoASH pool in strain NR1769 (ΔfabH) compared with WT (Fig. 7A). The major difference in the ACP thioester pool composition was also a large increase in malonyl-ACP in strain NR1769 (ΔfabH) compared with WT (Fig. 7B). The mass spectrometry data were normalized to either the [13C]acetyl-CoA (Fig. 7C) or [13C]acetyl-ACP (Fig. 7D) internal standards. The abundance of each intermediate was compared with the WT strain NR754. A 32-fold elevation in malonyl-CoA was the most notable feature in the ΔfabH cells (Fig. 7C). Complementation with FabH normalized the malonyl-CoA pool. Complementation with MadB also restored CoASH amounts, but there was a 4-fold decrease in the abundance of malonyl-CoA compared with WT. The relative abundance

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measurements showed that the ΔfabH deletion triggers a 64-fold increase in malonyl-ACP and a 16-fold decrease in butyryl-ACP, the only acyl-ACP intermediate dependent on FabH activity (Fig. 7D). Complementation with FabH normalized the malonyl-ACP abundance and also led to a substantial increase in butyryl-ACP. Complementation with MadB also normalized malonyl-ACP levels but led to a substantial increase in acetyl-ACP rather than butyryl-ACP (Fig. 7D). These measurements confirm the role of MadB as a malonyl-ACP decarboxylase in vivo. The ΔfabH mutation also led to substantially lower amounts of every acyl-, trans-2-enoyl-, and 3-hydroxyacyl-ACP intermediate in the cycle (Fig. S5). Complementation with FabH elevated these intermediates to higher levels than seen in WT cells showing that increased FabH initiation elevated FASII intermediates. MadB complementation restored the acyl-ACP intermediate levels to normal WT abundance. These data provide direct in vivo evidence that MadB is a malonyl-ACP decarboxylase and illustrate how Mad protein expression modifies the composition of the acyl-ACP pool.

**Acetyl-ACP is a GlmU substrate**

GlmU is a bifunctional enzyme that converts glucosamine-1-phosphate to UDP-GlcNAc (36–38). UDP-GlcNAc is required for the initiation of LPS (LpxA) (39, 40), PG (MurA) (41, 42) and the ECA (WecA) (43). Because of its central position in cell-wall formation, GlmU is under development as an antibiotic target (44). GlmU, like MadA, was scored as an ACP-binding partner in a protein-protein interaction screen (45), suggesting that acetyl-ACP may be an *E. coli* GlmU substrate. GlmU has been characterized using acetyl-CoA as the acetyl donor and exhibits a relatively high KM for acetyl-CoA (KM = 320–650 μM) (36, 46, 47). We purified GlmU (Fig. S6A) and developed an assay (Fig. S6B) to determine if acetyl-ACP is a substrate. The binding of acetyl-ACP (Fig. 8A) and acetyl-CoA (Fig. 8B) to GlmU was assessed by surface plasmon resonance. These experiments showed GlmU-bound acetyl-ACP with 10-fold higher affinity than acetyl-CoA.

GlmU is usually assayed by the spectrophotometric detection of the released CoA thiol (36, 46, 47) in 0.5 to 1 ml reactions, but a more sensitive assay is required to examine its affinity for a protein substrate. We developed an assay for the GlmU acetyltransferase half reaction in a 20 μl volume. The [14C]acetyl-ACP substrate and the N-[14C]acetylglucosamine-1-phosphate product were separated by thin-layer chromatography (Fig. S6B). The apparent KM for [14C]acetyl-ACP was 20 μM (Fig. 8C) compared with 400 μM for acetyl-CoA (Fig. 8D). These data identify acetyl-ACP as a high affinity *E. coli* GlmU substrate.

**Discussion**

This work identifies the members of Pfam_09500 as malonyl-ACP decarboxylases and define the role of MadA and acetyl-ACP in *E. coli* metabolism (Fig. 9). The Mad proteins are primarily expressed in Proteobacteria and come in two flavors: a GNAT-domain fused to a hot dog domain (MadA) or a standalone hot dog dimer (MadB). These enzymes produce acetyl-ACP that was first detected as a component of the ACP
thioester pool decades ago based on metabolic labeling and electrophoretic analyses in *E. coli* (5, 9). More recent mass spectrometry methods confirm acetyl-ACP is a major component of the *E. coli* ACP pool (18, 19). The origin of the acetyl-ACP pool has been a mystery, and the discovery of MadA identifies an enzyme responsible for acetyl-ACP synthesis in Proteobacteria. FabH is the major initiation enzyme that condenses acetyl-CoA with malonyl-ACP to form acetoacetyl-ACP that enters the elongation cycle at the FabG step (3-ketoacyl-ACP reductase) (5, 6) (Fig. 9). Mad proteins bypass FabH by converting malonyl-ACP to acetyl-ACP that is used by FabB/F to form acetoacetyl-ACP (2–4), which like the FabH product, enters FASII at the FabG step (Fig. 9). *S. oneidensis* has four genes that encode FabH enzymes (48). FabH1 and FabH2 initiate straight- and branched-chain FASII, respectively, and the other two genes are dispensable (48). The quadruple knockout still grows, albeit at a much slower rate, illustrating the presence of yet another mechanism to initiate FASII. *S. oneidensis* MadB characterized in this study is a likely candidate for the FabH bypass pathway in this organism.

A second advance from this study is the identification of acetyl-ACP as a high affinity GlmU substrate (Fig. 8). GlmU is an essential enzyme that produces UDP-GlcNAc, a substrate required for the initiation of key cell-envelope components: LPS (LpxA) (39, 40), PG (MurA) (41, 42) and the ECA (WecA) (43) (Fig. 9). Because of its central importance in the synthesis of cell wall polysaccharides, GlmU has received considerable attention as an antibiotic drug-discovery target (44). Previous research has used acetyl-CoA as the GlmU substrate (36, 46, 47). Acetyl-CoA is indeed a substrate for GlmU, but our work shows that acetyl-ACP is a much higher affinity substrate for the enzyme. These data suggest that UDP-GlcNAc may be a major route for acetyl-ACP metabolism in *E. coli* (Fig. 9). It is not clear how widespread acetyl-ACP usage by GlmU is in the bacterial kingdom because the Mad family of proteins are restricted to the Proteobacteria. GlmU is a key enzyme in cell wall biogenesis in all bacteria, which either indicates that acetyl-ACP does not support GlmU activity in most bacteria or that a different gene family encodes a malonyl-ACP decarboxylase in these bacteria. Thus, the Mad proteins supply acetyl-ACP for the initiation of the biosynthetic pathways for four major cell envelope components in Proteobacteria (Fig. 9).

The discovery of a family of malonyl-ACP decarboxylases identifies a new player in cell envelope biosynthesis in Proteobacteria, but many questions remain to be addressed. MadA is not an essential enzyme, therefore more experimental work will be required to understand the physiological
setting(s) where Mad dependent acetyl-ACP formation is beneficial or of regulatory significance. The intracellular levels of acetyl-CoA vary by an order of magnitude on different carbon sources (49, 50), suggesting that MadA may play a more important role in nutrient-limiting conditions. It is possible that acetyl-ACP may be a substrate in other unidentified reactions. Mad proteins appear confined to Proteobacteria, suggesting that acetyl-ACP formation may be most important in supporting fatty acid synthesis for LPS, an essential outer membrane constituent confined to this group of bacteria. The role of the amino terminal domain of the MadA remains to be elucidated. It is not required for malonyl decarboxylase activity and is not present in the majority of Mad genes in Proteobacteria. The MadAN configuration is confined to γ-Proteobacterial species, suggesting it has a specific function in these organisms. MadAN and MadAC are clearly independently folded protein domains opening the possibility that standalone GNAT enzymes carry out the MadAN function in Proteobacteria that express MadB. There are many candidates for a MadAN-like protein among the GCN5-related N-acetyl transferases of unknown function that are present in all bacterial genomes. We have not documented any impact of the MadAN domain on MadAC decarboxylase activity so perhaps MadAN carries out the acetylation of lysine residues on an unidentified target protein, as suggested by its homology to known GNAT N-acetyl transferases.

**Experimental procedures**

**Materials**

Malonyl CoA, NADPH, acetyl-CoA, magnesium chloride, sodium chloride, SYPRO orange gel stain, thrombin, glucosamine-1-phosphate, and monoclonal anti-His6 alkaline phosphatase conjugated antibody were purchased from MilliporeSigma. [1-14C]Acetyl-CoA (specific activity 60 mCi/mmol) and [2-14C]malonyl CoA (specific activity 55 mCi/mmol) were purchased from PerkinElmer and American Radiolabeled Chemicals, respectively. [U-13C]acetyl-CoA was from MilliporeSigma. Thrombin (Sigma T4648), streptavidin agarose (Thermo 20359), HPDP-biotin (Thermo 21341), and glucoseamine-1 phosphate (Sigma G9753) are used in this study. The bacterial media was purchased from BD Medical Technologies. Antibiotics, nickel resin, and DTT were purchased from GoldBio. Protease inhibitor and Cytiva Amersham ECF Substrate for Western Blotting were purchased from Thermo Fisher. Bacterial strains (Table S5) and plasmids (Table S6) are used in this study.

**Protein purification**

The YiiD (MadA) sequence from *E. coli* (Uniprot ID: P0ADQ2) and the domains MadAN and MadAC were amplified from Top10 genomic DNA and cloned by Gibson Assembly into pET28a (cut with NdeI + HindIII) under control of an IPTG inducible promotor to create plasmids pPJ604, pPJ605, and pPJ606. The sequence for MadAN included amino acids 1 to 161, and the sequence for MadAC included amino acids 171 to 329. A PCR product of the *E. coli* GlmU gene was cloned into NdeI-BamHI site of pET28a using Gibson Assembly to obtain pPJ602 and purified, as described (47). The DNA sequence of MadB (Uniprot ID: Q8E989) was cloned into the pET28a expression vector under control of an IPTG inducible promotor to create plasmid pPJ604, pPJ605, and pPJ606. The sequence for MadAN included amino acids 1 to 161, and the sequence for MadAC included amino acids 171 to 329. A PCR product of the *E. coli* GlmU gene was cloned into Ndel-BamHI site of pET28a using Gibson Assembly to obtain pPJ602 and purified, as described (47). The DNA sequence of MadB (Uniprot ID: Q8E989) was cloned into the pET28a expression vector under control of an IPTG inducible promotor to create plasmid pPJ612. The expression vectors for mutant MadB proteins were constructed by site-directed mutagenesis using the QuikChange Lightning kit.

All the proteins were expressed using pET expression vectors in *E. coli* BL21(DE3) cells. The constructs for ACP, FabH, FabD, and FabG have been described previously (13). Protein expression was induced with IPTG for 3 h at 37 °C (ACP,
Malonyl-ACP decarboxylase

FabH, FabD, FabG, and GlmU) or overnight at 16 °C (MadA, MadAN, MadA\textsuperscript{C}, MadB, and MadB mutants). After cell lysis, the protein was purified using nickel affinity chromatography. The pooled purified protein was either dialyzed overnight (ACP, FabH, FabD, FabG, and GlmU) or further purified by size-exclusion chromatography (MadA, MadAN, MadA\textsuperscript{C}, MadB, and MadB mutants). The His\textsubscript{6}-tag was then cleaved from ACP and MadAC by thrombin digestion and separated from the purity with 1 mM IPTG. The protein was purified by nickel affinity chromatography. GlmU was expressed in BL21(DE3) for 3 h at 37 °C after induction with 1 mM IPTG. The protein was purified, as described [47]. His-tagged GlmU was dialyzed overnight in Amicon Ultra centrifugal filters and quantified by UV absorption with the exception of ACP, which was quantified by the BCA protein assay kit (Pierce).

The protein expressed from the MadA gene resulted in two bands on SDS gel electrophoresis. The second band was slightly lower and less intense than the band running at the expected location, indicating a second start site in the construct. The sequence analysis revealed a methionine at position 17 of the annotated protein sequence that had an apparent ribosome-binding site upstream. Therefore, we used site directed mutagenesis of plasmid pPJ604 (QuikChange Lightning kit; Agilent) to create the MadA(M17A) mutant that purified as a homogenous protein. The MadA(M17A) mutant had the same thermal stability and enzyme activities as MadA. The experiments were performed using the MadA(M17A) construct.

Enzyme assays

Condensing enzyme activity of FabH and MadA was assayed in a reaction mixture containing: 0.1 M Tris, pH 7, 0.2 M NaCl, 30 μM ACP, 0.1 mM NADPH, 0.05 μg FabD with or without 0.5 μM FabG. In reactions following the acetyl-CoA carbon, 35 μM malonyl-CoA and 30 μM [\textsuperscript{14}C]acetyl-CoA were added, and in reactions following the malonyl-CoA carbon, 30 μM acetyl-CoA and 35 μM [\textsuperscript{14}C]malonyl-CoA were added. The ACP was reduced with 0.25 mM DTT before the other reaction components were added. The reactions were initiated by the addition of 1 nM FabH or 5 nM MadA. After incubation at 37 °C for 12 min, the reaction mixtures were centrifuged to remove any precipitated protein. CoA thioesters were quantified using a liquid chromatography (HPLC) method, as previously described [51].

Synthesis of labeled acyl-ACP

\textsuperscript{13}C- and \textsuperscript{14}C-labeled acyl-ACPs were synthesized by the previously described method for crotonyl-ACP synthesis [26]. The [U-\textsuperscript{13}C]acetyl-ACP reaction mixture contained the following: 300 μM purified ACP with 2 μM E. coli ACP synthase and 600 μM [U-\textsuperscript{13}C]acetyl-CoA in 0.05 M Tris, pH 7, with 0.01 M MgCl\textsubscript{2}. The [1-\textsuperscript{13}C]acyl-ACP reaction contained the following: 300 μM purified ACP with 4 μM E. coli ACP synthase, 300 μM acetyl-CoA, and 150 μM [1-\textsuperscript{13}C]acetyl-CoA in 0.1 M NaPO\textsubscript{4}, pH 7, with 0.01 M MgCl\textsubscript{2}. The [2-\textsuperscript{13}C]malonyl-ACP reaction contained the following: 300 μM purified ACP with 4 μM E. coli ACP synthase, 800 μM malonyl-CoA, and 400 μM [2-\textsuperscript{13}C]malonyl-CoA in 0.1 M Tris, pH 8.5, with 0.01 M MgCl\textsubscript{2}. All the reactions were incubated for 2 h at 37 °C. The His\textsubscript{6}-tagged [ACP]synthase was removed from the reactions by nickel affinity chromatography. The MgCl\textsubscript{2} and excess CoA were removed using a PD-10 desalting column. The labeled acyl-ACPs was eluted in 0.02 M Bis-Tris, pH 6, with 0.2 M NaCl and concentrated using Amicon Ultra centrifugal filters (3 kDa cut-off). The concentrated standards were quantified using the BCA protein assay kit (Pierce).

Thermal stability assays

Thermal stability of purified proteins was determined by monitoring protein binding to a fluorescent dye with
increasing temperature using an Applied Biosystems 7500 Fast Real Time PCR System according to previously published protocols (52, 53). The samples containing 10 μM protein in 0.05 M NaPO₄, pH 7, 0.3 M NaCl, and 2.5μ SYPRO Orange dye were loaded into 96 well ThermoGrid plates (Denville Scientific) and sealed. The plates were centrifuged at 1000g for 5 min. The samples were heated from 25 °C to 95 °C at 1 °C/min with a fluorescence measurement taken at each degree increase using a TAMRA filter set (Ex 560 nm and Em 582 nm). The resulting data was fit to the first derivative of the Boltzman sigmoidal equation using GraphPad/Prism software to determine the point at which 50% of the protein was denatured. The experiments were completed in triplicate.

Thermal stability of the MadB mutants was also determined by nanoDSF. Dynamic light scattering of the proteins was measured with the Prometheus NT.48 (NanoTemper Technologies). Briefly, 20 μl samples containing 20 mM Tris, pH 7.5, 0.2 M NaCl, 10 mM EDTA, and 10 μM MadB mutant protein were mixed and loaded into Prometheus NT.48 Series nanoDSF Grade High Sensitivity Capillaries (NanoTemper Technologies) by capillary force action. The capillaries were heated from 20 °C to 90 °C at a rate of 1 °C/min, and the UV light scattering was recorded to determine the Tagg.

Analytical ultracentrifugation

Sedimentation velocity experiments were conducted in a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter) after standard protocols unless mentioned otherwise (54). The samples in buffer containing 0.02 M Tris, pH 7.5, and 0.5 M NaCl were loaded into cell assemblies comprising of double sector charcoal-filled centerpieces with a 12 mm path length and sapphire windows. The buffer density and viscosity were calculated using the software SEDNTERP (http://www.jphilo.mailway.com/download.htm) (55). The partial specific volumes and the molecular masses of the proteins were calculated based on their amino acid compositions in SEDFIT (https://sedfitsetphat.nibib.nih.gov/software/default.aspx).

The cell assemblies, containing identical sample and reference buffer volumes of 390 μl, were placed in a rotor and temperature equilibrated at rest at 20 °C for 2 h before it was accelerated from 0 to 50,000 rpm. Rayleigh interference optical data were collected at 1-min intervals for 12 h. The velocity data were modeled with diffusion-deconvoluted sedimentation coefficient distributions in SEDFIT (https://sedfitsetphat.nibib.nih.gov/software/default.aspx), using algebraic noise decomposition and with signal-average frictional ratio and meniscus position refined with nonlinear regression (56). The s-values were corrected for time, and finite acceleration of the rotor was accounted for in the evaluation of Lamm equation solutions (57). Maximum entropy regularization was applied at a confidence level of P-0.70.

Homology modeling

MadA homology models were generated using SWISS-MODEL (58), using the S. aureus putative acetyltransferase SACOL1063 (PDB: 5Q4) and S. oneidensis thioesterase (PDB: 1T82) structures as templates for the amino (19–162) and carboxy (171–314) terminal domains, respectively. The theoretical MadA conformer domain assemblies were arranged in PyMOL (59).

Phylogeny

All sequences containing the Mad domain (PF_09500) in the Pfam protein database were aligned using MAFFT (60). A logo for the active site loop was created from this alignment using the online Seq2Logo tool (61).

Complementation strain construction

For the in vivo complementation experiments, FabH and Mad proteins were expressed in strain NR1769 (∆fabH). The sequences encoding His-tagged versions of FabH, MadA, MadAN, MadAC, and MadB were inserted into the pBAD vector (Thermo Fisher), which contains an arabinose inducible promoter. The plasmids were transformed into strain NR1769 by electroporation, and the positive transformants were selected by plating on kanamycin and carbenicillin.

Protein expression of complementation strains was confirmed by Western blotting. The overnight cultures were cut back to an optical density of 0.05 in LB and grown for 2 h at 37 °C with shaking. A₆₀₀ were adjusted to 0.05 in Luria broth with 0.1% arabinose to induce expression of His₆-tagged proteins from pBAD vectors. The cultures were incubated 75 min at 37 °C with shaking. The cell pellets were collected by centrifugation and stored at −80 °C. The pellets were resuspended in 0.02 M Tris, pH 7.9, 0.5 M NaCl, 10% glycerol, and 0.01 M imidazole with protease inhibitor and lysed in a French press. The samples were centrifuged at 20,000g for 30 min at 4 °C to remove insoluble debris, and the protein was quantified using BCA assay and equal amounts of protein were separated on a 10% Bis-Tris gel. The protein was transferred to a polyvinylidene difluoride membrane using the iBlot 2 Dry Blotting System (Thermo Fisher). The membrane was incubated in 5% milk for 1 h at RT, then overnight at 4 °C in a 1:2000 dilution of anti-His₆ alkaline phosphatase-conjugated monoclonal antibody in 5% milk. The membrane was washed in Tris buffered saline with 0.1% Triton X-100 for 15 min intervals for a total of 1 h. ECF substrate was added for 1 to 3 min, and the membrane was imaged using the Typhoon 9200 Phospholmager.

Doubling time and cell size

Doubling time and cell size estimation experiments were performed according to previously published methods with modifications (11, 12). The overnight cultures were diluted to an A₆₀₀ of 0.05 in LB and allowed to incubate at 37 °C for 2 h. The cultures were diluted to 0.05 again, and 0.1% arabinose was added. The cultures were incubated at 37 °C for 6 h with aliquots removed at 20 min intervals to record the A₆₀₀. The doubling times were determined by plotting A₆₀₀ versus time in GraphPad/Prism and fitting the linear portion of the curve to the exponential growth equation. The experiments were performed in triplicate.
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The samples for cell size estimation were removed from the above growth experiments when cultures reached an A_{600} of 0.2 to 0.4. One ml of the culture was centrifuged at 13,000 rpm for 1 min. The supernatant was removed, and the cells were resuspended in 1 ml 4% formaldehyde in phosphate buffered saline, pH 7. The cells were incubated for 15 min at RT followed by 15 min on ice and then washed twice with PBS. The cells were then resuspended in a final volume of 25 μl PBS. Two μl of the cells was spotted onto a coverslip and allowed to dry. The coverslips were mounted with 15 μl 50% glycerol in PBS and allowed to dry for at least 1 day. Differential Interference Contrast imaging was performed using a 100× objective on a Nikon Eclipse Ni Widefield Microscope. The length and width measurements of 50 dividing cells with a figure “8” morphology for each strain were recorded using Nikon NIS-Elements imaging software.

Acyl-CoA quantification

Overnight cultures of strains NR1769/pBAD, NR1769/pFabH, and NR1769/pMadB were diluted to A_{600} = 0.05 in M9 medium with 1 mM MgSO_4, 0.1 mM CaCl_2, and 0.4% glucose. After 2 h, the incubation at 37 °C cultures were diluted to A_{600} = 0.05 in the same media and supplemented with 0.1% arabinose to induce expression of FabH or MadB. The cultures were incubated at 37 °C until they reached an A_{600} = 0.6. The extraction method, liquid chromatography column and gradient, mass spectrometry parameters, and the Q1 and Q3 masses for the acyl-CoA were exactly as described (17).

Acyl-ACP quantification

The samples were prepared for acyl-ACP quantification, as previously described (17, 18). One milliliter aliquots of cultures at mid-log phase growth were removed to a 1.5 ml Eppendorf tube containing 250 μl ice cold 10% trichloroacetic acid. The tubes were inverted and centrifuged to collect the precipitate that was washed with acetone, dried, and stored at −80 °C. The pellets were resuspended in 100 μl of lysis buffer (0.05 M NaPO_4, pH 7.2, 1 mM ascorbic acid, 2 mM ethylendediaminetetraacetic acid, and 6 M urea) prepared just before use. [1,13C]Acetyl-ACP was added as an internal standard (1 μl of 0.0125 μg/μl [U-13C]Acetyl-ACP). A chloroform/methanol extraction was performed by adding 400 μl methanol, followed by 100 μl of chloroform. The samples were sonicated in a sonication bath (Fisher Scientific CPXH Series 1.9L) for 10 min at room temperature to resuspend. The phases were separated by adding 300 μl of 200 mM formate buffer (pH 3.9). The upper phase was discarded, and 300 μl methanol was added to the remaining sample. The precipitated proteins were washed with 300 μl of methanol and then dried. ACP was resuspended in 10 μl of 0.1 M NaPO_4, pH 6.5, and sonicated for 10 min at room temperature in an ultrasonic bath. Insoluble debris was pelleted, and 5 μl of resuspended ACP was removed to a clean tube. ACP was digested by adding 0.5 μg endoproteinase Asp-N (MilliporeSigma) in 10 μl of 0.1 M Tris, pH 7.5 (62). The samples were incubated 1 h at room temperature to allow for complete digestion with minimal degradation of acyl chains. Proteolysis was quenched with 15 μl methanol. The liquid chromatography column and solvent gradient, mass spectrometry parameters, and Q1 and Q3 masses for acyl-ACPs were exactly as described (17).

GlmU assay

GlmU assays were performed with [1-14C]Acetyl-CoA and [1,14C]Acetyl-ACP. ACP in pET15b was expressed in BL21(DE3) and purified with Ni2+-NTA affinity chromatography. The His-tag from the His_{6}-ACP was cleaved using thrombin overnight at room temperature, and the reaction was precipitated using 1% TCA. The pellet was resuspended in 20 mM Bis-Tris, pH 6.5 and centrifuged to remove any precipitated protein that did not go into solution. A Zebap spin column (Thermo Fisher) was used to exchange the buffer to 20 mM Tris, pH 7.5. DTT was added to a final concentration of 10 mM and incubated at 37 °C for 1 h to reduce the contaminating ACP; the excess DTT was removed using a Zeba spin column, and the buffer was changed to PBS containing 1 mM EDTA and 250 mM NaCl. To remove ACP, the eluate was mixed with HPDP-Biotin for 2 h at room temperature, and a Zeba spin column was used to remove any unreactive HPDP-Biotin. Streptavidin agarose was used to remove ACP bound to HPDP-Biotin; the supernatant contained pure apo-ACP was then heated at 95 °C for 10 min to destroy contaminating ACP-binding proteins. This step was essential to remove GlmU (and other FASII proteins) from the preparation. [1-14C]Acetyl-CoA (specific activity 60 mCi/mmol) was used to generate [14C]acyetyl-ACP using [ACP] synthase, briefly the reaction mixture containing 100 mM Tris, pH 7.0, 10 mM MgCl_2, 300 μM apo-ACP, 400 μM [1,14C]acetyl-CoA, and 4 μM [ACP]synthase was incubated at 37 °C for 2 h. Ni2+-NTA was added to remove the His-tagged [ACP] synthase, and the buffer was exchanged using a PD-10 column to 20 mM Bis-Tris, pH 6.0, and 200 mM NaCl. Radioactive GlmU assay contained the following: 20 mM KH_2PO_4, 20 mM MgCl_2, 0.1% BME, 500 μM glucoseamine-1-phosphate (GlcN-1P), [14C]Acetyl ACP (0–32 μM), and 0.6 ng of GlmU. The reaction was incubated for 15 min at 37 °C, and 10 μl of the reaction was spotted on a Silica Gel H plate and developed with 1-butanol:methanol:ammonia:water (5:4:2:1, v/v/v/v). The reaction with [14C]Acetyl-CoA (0–320 μM) (specific activity 20 mCi/mmol) was performed similarly but with 3 ng of GlmU. The bands on the plate were quantified using a Typhoon 9200 PhosphoImager and ImageQuant. The experiment was repeated three times, and the data were fit to one site binding Hill equation (GraphPad/Prism software).

Surface plasmon resonance

The experiments were conducted at 20 °C using a Pioneer optical biosensor (Sartorius). His-tagged E. coli GlmU was immobilized on a polycarboxybetate hydrogel-coated gold chip preimmobilized with nitrioltriatic acid (His Cap chip; Sartorius) by capture-coupling, a hybrid method of capture and amine coupling chemistry (63). The chip was primed in chelating buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 50 μM
EDTA, and 0.005% Tween 20) and was preconditioned at 10 μl/min with three 60 s injections of wash buffer (10 mM Hepes, pH 8.3, 150 mM NaCl, 350 mM EDTA, and 0.05% Tween-20) and one 60 s injection of chelating buffer before being charged with a 60 s injection of 500 μM NiCl2 in chelating buffer. The charged chip was primed with immobilization buffer (10 mM Hepes pH 7.5, 300 mM NaCl, 1 mM TCEP, 10% glycerol, and 0.005% Tween 20), and carboxyl groups on the hydrogel were activated with N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. GlmU was injected over the activated surface to dimensions Dmax was determined from the pair distance distribution function P(r) with the program GNOM (65, 66). Twenty reconstructions were aligned, and all were averaged and refined in slow mode using DENSs (32) to calculate the electron density for the final 3-D reconstruction.

Data availability
All data are presented in the article.

Supporting information—This article contains supporting information.

Acknowledgments—We thank Pam Jackson and Karen Miller for expert technical assistance with strain construction, protein purification, and enzyme assays. Dr Natividad Ruiz kindly provided strains. Amanda Nourse, Brett Waddell, and Siva Vaithilingam from the St Jude Molecular Interaction Analysis shared resource provided analytical ultracentrifugation, surface plasmon resonance, and nanoDSF experiments, respectively. We also thank Srinivas Chakravarty for assistance with SEC-SAXS data collection and analysis, Ti-Cheng Chang for assistance with phylogenetic analysis, Chris Fiveash for graphic design, and the St Jude Cell & Tissue Imaging Center for assistance with microscopy data acquisition.


Funding and additional information—This research was supported by NIH grant GM034496 (C. O. R.). This research was also supported by the Cancer Center Support Grant CA21765 and the American Lebanese Syrian Associated Charities. The Advanced Photon Source is operated for the Department of Energy Office of Science by Argonne National Laboratory under Contract DE-AC02 to 06CH11357. The SEC-SAXS experiments were supported by NIH grant GM103622. Use of the Pilatus 3 M detector was provided by support from NIH grant OD018090. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Malonyl-CoA and malonyl-ACP stability
Stabilities of malonyl-ACP and malonyl-CoA were determined by incubating 10 μg malonyl-ACP or 4 mM malonyl-CoA in 0.02 M Bis-Tris, pH 6, and 0.2 M NaCl for 1 or 16 h at room temperature, 30 °C or 37 °C. After incubation, the malonyl-ACP samples were separated using a concentrationally sensitive urea gel (0.5 M urea, 13% acrylamide), stained with Coomassie dye and imaged using the Bio-Rad Gel Doc XR+ imaging system. After incubation, 40 μl of the methanol was added to 10 μl of each malonyl-CoA condition, and the CoA was quantified using the HPLC method previously described (51).

SEC-SAXS
SEC-SAXS experiments were performed at the BioCAT beamline 18-ID-D at the Advanced Photon Source. Photons that scattered from the λ = 1.033 Å X-ray beam were recorded on the Pilatus3 X 1M detector at a sample-to-detector distance of 3.631 m, accessing a range of momentum transfer (q) from 0.0047 to 0.35 Å−1. A Superdex 200 10/300 increase column was preequilibrated with 20 mM Tris, pH 7.5, 200 mM NaCl, 1% glycerol, and 10 mM EDTA. MadA (290 μl, 6.1 mg/ml) was injected onto the column with a flow rate of 0.6 ml/min that enabled the separation of potential aggregates before flowing MadA through a temperature-controlled quartz capillary (1.0 mm internal diameter) flow cell for X-ray exposure. SAXS was performed in-line with this size-exclusion chromatography setup. One peak of X-ray scattering was detected by 0.5 s SAXS exposures of fractions of the column recorded every second. Buffer background was obtained from a baseline region of the chromatogram and subtracted from the averaged central portion of the MadA peak to obtain the scattering profile. The data were reduced, processed, and the overall parameters computed following standard procedures of the software package RAW (64) version 2.0.3. A linear Guinier plot indicated there was no significant radiation damage during the exposure period (not shown). The zero-angle intensity I0, radius of gyration Rg, and associated uncertainties for these parameters were obtained by weighted linear regression of log(I) versus q2 as shown in the Guinier plot. The particle dimension Dmax was determined from the pair distance distribution function P(r) with the program GNOM (65, 66).

This research was supported in part by NIH grant OD018090. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ACP, acyl carrier protein; ECA, enterobacterial common antigen; FabH, 3-ketoacyl-ACP synthase III; FASII, type II bacterial fatty acid synthesis system; GlimU, glucosamine-1-phosphate N-acetyl transferase/N-acetylglucosamine-1-phosphate uridyltransferase; GNAT, GCN5-related N-acetyl transferase; Mad, malonyl-ACP decarboxylase; LPS, lipopolysaccharide; PG, peptidoglycan; SAXS, small angle X-ray scattering.

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