Noncanonical coproporphyrin-dependent bacterial heme biosynthesis pathway that does not use protoporphyrin

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It has been generally accepted that biosynthesis of protoheme (heme) uses a common set of core metabolic intermediates that includes protoporphyrin. Herein, we show that the Actinobacteria and Firmicutes (high-GC and low-GC Gram-positive bacteria) are unable to synthesize protoporphyrin. Instead, they oxidize coproporphyrinogen to coproporphyrin, insert ferrous iron to make Fe-coproporphyrin (coproheme), and then decarboxylate coproheme to generate protoheme. This pathway is specified by three genes named *hemY*, *hemH*, and *hemQ*. The analysis of 982 representative prokaryotic genomes is consistent with this pathway being the most ancient heme synthesis pathway in the Eubacteria. Our results identifying a previously unknown branch of tetrapyrrole synthesis support a significant shift from current models for the evolution of bacterial heme and chlorophyll synthesis. Because some organisms that possess this coproporphyrin-dependent branch are major causes of human disease, HemQ is a novel pharmacological target of significant therapeutic relevance, particularly given high rates of antimicrobial resistance among these pathogens.

heme synthesis | Gram-positive bacteria | HemQ | coproporphyrin | HemN

Many biological processes depend upon heme, an iron-containing porphyrin (1, 2). This prosthetic group is essential for the function of diverse proteins, including cytochromes, globins, peroxidases, catalases, and sensors that bind diatomic gases. Moreover, heme affects multiple aspects of bacterial pathogenesis, such as the ability of *Mycobacterium* and *Campylobacter* to scavenge reactive nitrogen species produced by host immune systems (3, 4) and the ability of *Staphylococcus* to modulate virulence (5, 6). Although the ability to synthesize heme is not ubiquitous in distribution, there are few organisms that do not synthesize heme and even fewer that lack heme altogether.

For over 50 y, it has been commonly accepted that the metabolic intermediates in the heme biosynthetic pathway are conserved among all organisms, with the one variation being the manner by which the first committed intermediate, 5-aminolevulinate (ALA), is synthesized (1, 7). Whereas the route for ALA synthesis that employs a single enzyme (HemA) using Gly and succinyl-CoA as substrates was the first to be described (often called the four-carbon or Shemin path), it is the glutamyl-tRNA–based synthesis described in the 1970s (often called the five-carbon path), which relies upon two enzymes, glutamyl-tRNA reductase and Glu-1-semialdehyde-2,1-aminomutase, that is most prevalent across all forms of life (7, 8). Alternative enzymes to accommodate aerobic vs. anaerobic lifestyles have been described for the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX (aerobic: HemF, anaerobic: HemN/CpdH) (7, 9, 10) and the oxidation of protoporphyrinogen IX to protoporphyrin IX (aerobic: HemY, mixed: HemG and HemJ) (7, 11, 12) (Fig. S1). However, in each of these instances, the metabolic intermediates in the pathway are invariable.

The first experimental hint that the "classic" protoheme synthetic pathway was not universal came in 1998 with the postulation of a "primitive" pathway in *Desulfovibrio vulgaris* (13). This pathway, named the "alternative heme biosynthesis" path (or *ahb*), has now been characterized by Warren and coworkers (15) in sulfate-reducing bacteria. In the *ahb* pathway, siroheme, synthesized from uroporphyrinogen III, can be further metabolized by successive demethylation and decarboxylation to yield protoheme (14, 15) (Fig. 1 and Fig. S1). A similar pathway exists for protoheme-containing archaea (15, 16).

Current gene annotations suggest that all enzymes for prokaryotic heme synthetic pathways are now identified. However, confounding issues exist. First, the validity of most *hemN* annotations for oxygen-independent coproporphyrinogen oxidases (also known as coproporphyrinogen dehydrogenase or decarboxylase) is questionable, and there is no biochemical or genetic evidence supporting the existence of a coproporphyrinogen oxidase enzyme in any of the Firmicutes or Actinobacteria (herein referred to as Gram-positive bacteria). Second, in Gram-positive bacteria, the protein HemQ, annotated as a member of the chlorite dismutase (Cld) family, has been shown to be necessary for heme synthesis (17), although no function has been ascribed to this protein (17, 18). Finally, protoporphyrin has never been identified as a pathway intermediate in Gram-positive bacteria (19, 20).

Herein, we show that Firmicutes, Actinobacteria, and several other bacterial taxa synthesize protoheme via a noncanonical pathway that is different from the pathways found in eukaryotes, Archaea, Proteobacteria, or sulfate-reducing bacteria (15, 16).

Significance

It has been accepted dogma that eukaryotes and heme-synthesizing bacteria use the same metabolic intermediates in their heme synthesis pathways, where protoporphyrin is the final intermediate into which iron is inserted to make protoheme. Herein, we present data demonstrating that Gram-positive bacteria do not use protoporphyrin as an intermediate but, instead, have an altered set of terminal reactions that oxidize coproporphyrinogen to coproporphyrin and insert ferrous iron into coproporphyrin, resulting in the formation of coproheme. A newly characterized enzyme, HemQ, decarboxylates coproheme to generate protoheme. Because some organisms that possess this coproporphyrin-dependent branch are major causes of human disease, HemQ is a novel pharmacological target of significant therapeutic relevance, particularly given high rates of antimicrobial resistance among these pathogens.

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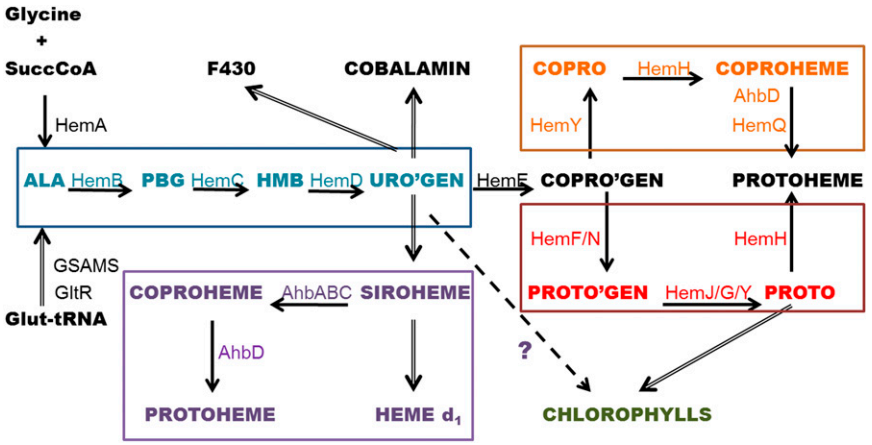


Fig. 1. Currently characterized tetrapyrrole biosynthesis pathways. The core enzymes present in all tetrapyrrole-synthesizing organisms are shown in the blue box. The protoporphyrin-independent, ancestral heme biosynthesis pathway of the Firmicutes and Actinobacteria described in the current study is shown in orange. The primitive heme pathway of the archaea and sulfate-reducing bacteria is shown in the violet box, and the modern heme pathway of Proteobacteria and eukaryotes is shown in the red box. Enzyme abbreviations are those abbreviations currently in acceptance and are shown with single solid lines. Pathways involving more than a single enzyme are shown as double-line arrows. The dashed line represents a proposed pathway for chlorophyll synthesis in bacteria that do not use protoporphyrin. ALA, 5-aminolevulinic acid; COPRO, coproporphyrin; COPRO'GEN, coproporphyrinogen; GltR, glutamyl-tRNA reductase; Glut-tRNA, glutamyl-tRNA; GSAMS, Glu-1-semialdehyde-2,1-aminomutases; HMB, hydroxymethylbilane; PBG, porphobilinogen; PROTO, protoporphyrin; PROTO'GEN, protoporphyrinogen; succCoA, succinyl-coenzymeA; URO'GEN, uroporphyrinogen.

Given that some of the Gram-positive bacteria that possess this pathway are pathogens and contributors to human disease, HemQ represents a novel pharmacological target of significant therapeutic relevance.

Results

Analysis of Current *hemN* Annotations. In the face of rapidly expanding genomic data, annotation has had to rely on an insufficiently small number of experimentally validated functional assignments (21). Unfortunately, this intrinsically error-prone process created substantial problems in the annotation of the *hemN/Z* branch of the radical *S*-adenosyl methionine (SAM) superfamily as all being oxygen-independent coproporphyrinogen oxidase. Using a comparative genomics approach to identify genes that encode coproporphyrinogen dehydrogenases (CpdHs), all genes annotated as oxygen-independent coproporphyrinogen oxidases (*hemN* and *hemZ*) were reexamined. This analysis was performed in the SEED database (22), and the results are presented in the SEED subsystem "Heme biosynthesis: Protoporphyrin- and coproporphyrin-dependent pathways" (23).

Based on genomic context and amino acid sequence comparisons, gene products fit into four distinct groups within the radical SAM enzyme superfamily (Table S1) and subsystem in SEED (23). The first of these groups, group A, represents the bona fide CpdH and contains the experimentally validated *Escherichia coli* HemN. Multiple sequence alignment using ClustalW (24) (Fig. S2) revealed that conserved regions correspond to those regions present in all radical SAM enzymes [e.g., the S-adenosyl methionine–binding sites and the Fe-S cluster motif (CXXXCXXC)]. However, only the group A family contains the motifs of *E. coli* HemN that are essential for CpdH activity: $_{20}$GPRYTSYPTA$_{29}$ and $_{308}$KNFQGYTT$_{315}$ (7, 10). Thus, these data suggest that only group A members possess the necessary structural attributes to be classified as CpdH. Group B members are exemplified by *Vibrio cholerae* HutW, a protein that does not possess CpdH/CpoX activity but appears to be involved in heme uptake and/or utilization (25). Group C members are found in a wide variety of organisms, including many incapable of heme biosynthesis. These group C genes are often clustered with those genes related to nucleotide metabolism. Finally, group D contains *hemNs* in the Firmicutes and Actinobacteria, including those annotated in *Streptococcus* species that do not synthesize heme. Thus, there exist no identifiable enzymes to produce protoporphyrinogen in many bacterial genomes (otherwise encoding a full complement of heme biosynthetic genes), especially in Firmicutes, Actinobacteria, Planctinomyces, and several other taxa (23) (Dataset S1).

Identification of the Terminal Enzymes of Gram-Positive Heme Synthesis. Our current bioinformatics analyses indicate that bona fide CpdH (HemN) is found in Proteobacteria, cyanobacteria, the Bacteroidetes/Chlorobi group, the Chlamydiae Chlamydiae/Verrucomicrobia group, and several other phyla, but it does not occur in any sequenced genomes of Firmicutes or Actinobacteria. Likewise, the aerobic form of coproporphyrinogen oxidase, HemF, is found in the same major taxa as HemN but is not present in sequenced genomes of Firmicutes or Actinobacteria (with very few exceptions). Extensive biochemical and bioinformatics approaches in our laboratory failed to identify a gene/protein in Gram-positive organisms that catalyzed the conversion of coproporphyrinogen to protoporphyrinogen. However, our screens did identify a protein of unknown function (HemQ) that is essential for heme synthesis in Firmicutes and Actinobacteria (17). These data and experiences led us to reevaluate the state of knowledge about heme synthesis in bacteria.

The *hemY* gene was originally identified as part of the *Bacillus subtilis* gene cluster *hemEHY* (26), and thereafter was shown to encode a protein that was capable of oxidizing protoporphyrinogen IX to protoporphyrin IX (27, 28). A crystal structure exists for this protein (29), which is of a similar size and structure as the eukaryotic PpoX (30, 31) and Gram-negative HemY (32). However, it possesses four distinctions from these enzymes: (*i*) it is a soluble monomer, (*ii*) it is relatively insensitive to the herbicide acifluorfen, (*iii*) it is unable to complement a Δ*ppoX* (*hemG*) mutant of *E. coli*, and (*iv*) it is able to oxidize coproporphyrinogen to coproporphyrin. Indeed, it oxidizes coproporphyrinogen at a rate almost ninefold faster than it oxidizes protoporphyrinogen (7.0 nmol·min^{-1} vs. 0.85 nmol·min^{-1}, respectively) and has a lower apparent K_m (0.56 μM vs. 0.95 μM, respectively) (28). Additionally, when *B. subtilis* HemY is overexpressed in *E. coli*, it causes the accumulation of coproporphyrin (27), not protoporphyrin. To justify the assignment of this HemY as a protoporphyrinogen (rather than a coproporphyrinogen)

oxidase, it has been proposed that, in vivo, it exists in a complex with other proteins where HemY would be prevented from encountering coproporphyrinogen. The possibility that HemY functions in vivo in Gram-positive bacteria to oxidize coproporphyrinogen to coproporphyrin had not been examined experimentally.

Ferrochelatase (HemH) from *B. subtilis* has been well characterized, including elucidation of its crystal structure at 1 Å (33, 34). HemHs from the Actinobacteria have also been identified and characterized, and they have been found to have a [2Fe-2S] cluster similar to what is found in metazoan ferrochelatases (34–36). Ferrochelatases are structurally well conserved, with the exception that the enzymes from Gram-positive organisms lack a small loop that forms one lip of the active site mouth (34) (Fig. S3). Interestingly this lip, in ferrochelatases that possess it, encloses the active site during catalysis, thereby forming a snug pocket that encloses the A and B rings (which possess vinyl groups at the 2, 4 position) of the macrocycle (37) (Fig. S1).

We examined the substrate specificity of ferrochelatase from *B. subtilis* (a Firmicute) and *Mycobacterium tuberculosis* (an Actinobacteria), along with human ferrochelatase, to determine if they could use the coproporphyrin that would be produced in vivo by HemY. All proteobacterial and eukaryotic ferrochelatases examined to date do not catalyze the insertion of iron into coproporphyrin at measurable rates. We find that the tested purified ferrochelatases from these representatives of both phyla use coproporphyrin at a rate that is greater than is found with protoporphyrin (Fig. S4 and Tables S2 and S3). Indeed, we determined that the K_m of *M. tuberculosis* ferrochelatase for coproporphyrin III (10.5 μM) is over 60-fold lower than its K_m for protoporphyrin IX (720 μM) and that the k_{cat} is 1.8 min^{-1} vs. 0.8 min^{-1} (Fig. S5). For *B. subtilis* ferrochelatase, we determined that the K_m for coproporphyrin III is 7.8 μM and that the k_{cat} is 0.11 min^{-1}. In our hands, the activity of *B. subtilis* ferrochelatase for protoporphyrin is too low to measure, although it has previously been reported that the K_m for protoporphyrin is 8.0 μM (38). Of note is that both I and III isomers of coproporphyrin serve as substrates for these enzymes (Fig. S4). The substrate specificity with regard to metals is not distinctly different from eukaryotic ferrochelatases (Tables S2 and S3).

Previously, we identified HemQ (COG3253) as an essential component of heme synthesis in Firmicutes and Actinobacteria (17). Our initial genomic analysis identified HemQ as being the best candidate for the missing coproporphyrinogen oxidase (17). However, we were unable to detect any coproporphyrinogen oxidase activity. Because *Propionibacterium acnes* HemH and HemQ exist as a fusion protein, one possibility was that HemQ served as a scaffold upon which HemY and HemH may assemble. However, the lack of an identifiable stable complex in vitro did not support this model. Since then, others have reexamined HemQ, corroborating our finding that it lacks coproporphyrinogen oxidase activity, and have also demonstrated that KO of *hemQ* in *Staphylococcus aureus* results in the accumulation of coproporphyrin (18).

The structure of three HemQs (annotated as Clds) has been determined and reveals that HemQ is a homopentamer (Protein Data Bank ID codes 1T0T, 1VDH, and 3DTZ). Unfortunately, the available protein structures of HemQs do not have bound product or substrate. For the HemQs we studied, the purified protein contains no metals or cofactors, but one can titrate protoheme into the apoprotein to produce a stable hemoprotein (17). With the realization that HemY + HemH of Gram-positive bacteria catalyze the formation of coproheme in vitro, we examined HemQ to determine if it converts coproheme to protoheme. Previously, we have shown that concurrent expression of HemY + HemH + HemQ in *E. coli* lacking either HemH or HemG resulted in rescue of these cells and that this occurs without the accumulation of coproporphyrin (17). When recombinant HemY + HemH + HemQ of *P. acnes* (or *M. tuberculosis*) were assayed in vitro with coproporphyrinogen and ferrous iron, the products observed were protoheme and an intermediate, which was identified by mass spectrophotometry as monovinyl, monopropionyl heme, resulting from the decarboxylation of a single propionate side chain (Fig. 2). The stereochemistry of the single decarboxylation (i.e., vinyl at the A or B ring) was not determined. When HemH and HemQ, together with coproporphyrin and ferrous iron, or HemQ alone with coproheme was assayed,

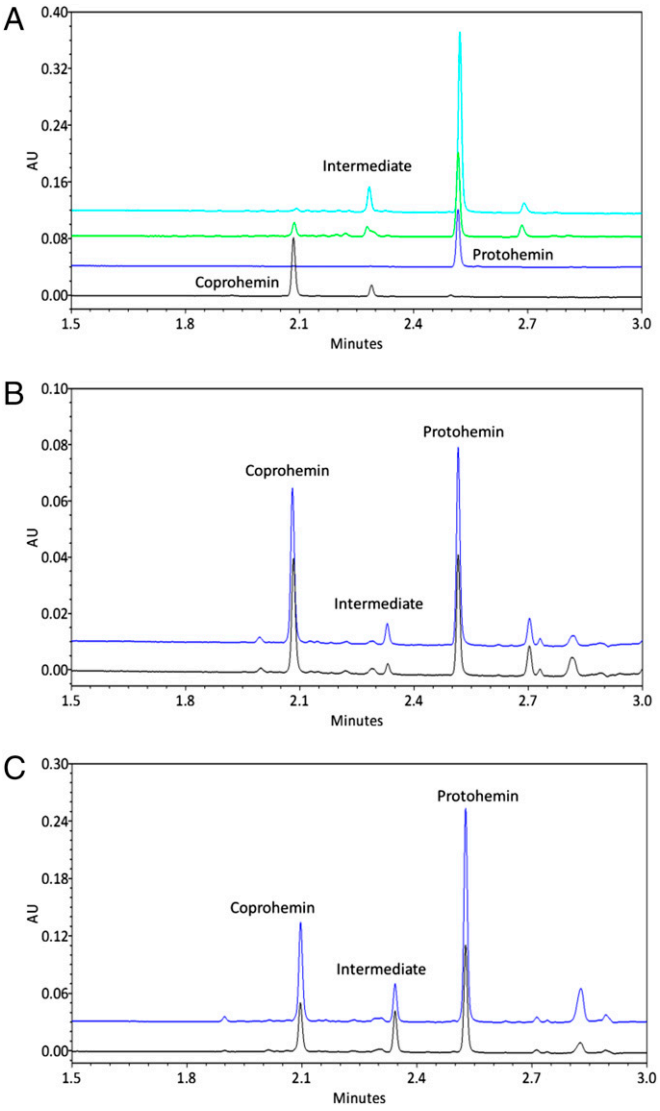


Fig. 2. HPLC chromatograms of reaction products of HemY/H^C/Q reactions. Assays were run as described in the main text. Elution time is shown on the *x* axis, and absorbance is shown on the *y* axis. (*A*) Reaction products from *P. acnes* HemY + HemH + HemQ with coproporphyrinogen and iron as substrates are shown in the first line (cyan). Reaction products from assay with coproporphyrin and iron as substrates with 50 μM FMN present are shown in the second line (green). Protohemin (blue) and coprohemin (black) standards are shown in the third and fourth lines, respectively. (*B*) Assay products from *M. tuberculosis* HemQ assayed with coprohemin as a substrate in the presence of 50 μM H_2O_2 (blue) or 50 μM FMN (black). (*C*) Assay products from *M. tuberculosis* HemQ that was not heme-loaded (blue) or was loaded with heme and then assayed with coprohemin as a substrate in the presence of 50 μM FMN. The mass spectrum results yielded 752.2 (coproheme + formate) for peak 1, 706.2 (monovinyl, monopropionyl heme intermediate + formate) for peak 2, and 660.2 (protoheme + formate) for peak 3.

protoheme was identified as the product, although at low concentrations. Under identical conditions, however, HemQ did not convert Ni-coproporphyrin into Ni-protoporphyrin.

The oxidative decarboxylation of coproheme to protoheme generates four protons, along with two CO_2s , and requires an electron acceptor. Because HemQ has been noted to have low peroxidase activity (17, 18), the possibility that HemQ was acting as a decarboxylase/peroxidase was examined. Inclusion of H_2O_2 in the reaction of HemQ with coproheme resulted in production of protoheme (Fig. 2*B*). However, the overall amount of coproheme + protoheme present after the assay period was less than expected, suggesting that the product heme was being degraded in the presence of peroxide. This finding is consistent with previous observations that "holo-HemQ" degrades its heme in vitro (18) and suggests that in the absence of a heme acceptor, HemQ-bound heme may be degraded. Some common electron acceptors were examined for their ability to stimulate the reaction. NAD, NADP, or FAD at a final concentration of 50 μM had no impact on protoheme production by HemQ, but inclusion of FMN resulted in conversion of coproheme into protoheme with HemQ alone (Fig. 2*B*). Of note is that inclusion of FMN had no measurable impact on protoheme formation by HemY + HemH + HemQ from coproporphyrinogen + iron. Because HemQ is known to bind both ferrous and ferric iron-protoporphyrin, the possibility that a protoheme HemQ "holoenzyme" is the actual decarboxylase rather than a product-bound form of the enzyme was examined. Coproheme decarboxylase assays with either heme-free or heme-bound HemQ were carried out, and there was no distinguishable difference between them (Fig. 2*C*).

Genomic Analysis of Heme Synthesis in Firmicutes and Actinobacteria. Our findings demonstrate that HemY, HemH, and HemQ in these organisms effectively work to oxidize coproporphyrinogen to coproporphyrin, insert ferrous iron to make coproheme, and then decarboxylate the 2, 4-position propionates of coproheme in a stepwise fashion to form the vinyl groups of protoheme (Fig. 3). Thus, for the Gram-positive organisms selected for biochemical characterization in this study, we have defined a new pathway for protoheme synthesis that does not involve protoporphyrin as an intermediate.

To avoid unwarranted generalization of our data, we have used a genomic analysis approach to identify and compare the

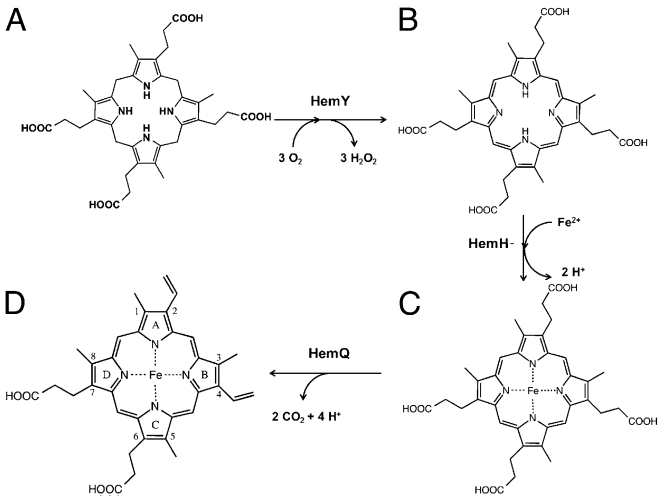


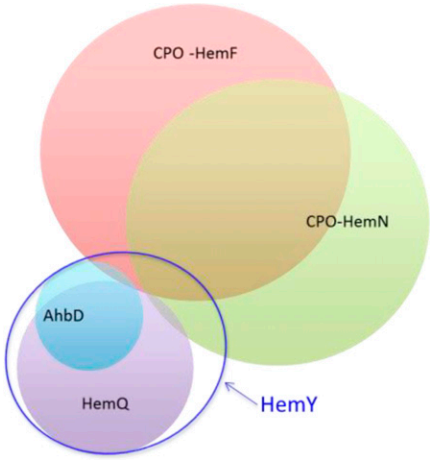
Fig. 3. Reaction catalyzed by the enzymes HemY, HemH, and HemQ (details are provided in main text). Coproporphyrinogen III (*A*), coproporphyrin III (*B*), coproheme III (*C*), and protoheme IX (*D*) are shown. Pyrrole ring lettering and side-chain numbering are shown on protoheme IX.

existence of all known bacterial enzymes that catalyze the oxidative decarboxylation of the 2, 4-position propionates of the coproporphyrin(ogen) tetrapyrrole-to-vinyl groups. This list includes HemF, HemN, HemQ, and AhbD. The data are presented in tabular (Dataset S1) and diagrammatic (Fig. 4) forms. HemF and HemN are present in Proteobacteria, cyanobacteria, the Bacteroidetes/Chlorobi group, the Chlamydiae/Verrucomicrobia group, Aquificae, Gemmatimonadetes, and several other taxa, and they overlap significantly with each other. HemQ is distinct from HemF/N, and it is found in nearly every Firmicute and Actinobacteria with currently sequenced genomes, and also in evolutionarily early-branching (Acidobacteria and *Planctomyces*) and transitional (*Deinococcus-Thermus* group) diderm phyla (39, 40). HemQ is the sole coproheme decarboxylase in almost 70% of genomes of Gram-positive bacteria (Fig. 4, Fig. S6, and Dataset S1). However, in ∼30% of the genomes possessing HemQ, it coexists with a clear homolog of AhbD in the absence of other enzymes of the siroheme-to-protoheme pathway (AhbA, AhbB, and AhbC). Indeed, AhbD is found to co-occur more frequently with HemQ (along with HemY and HemH) than it does with AhbA, AhbB, and AhbC. Among genomes that possess AhbD, ∼60% contain HemQ and do not contain AhbA, AhbB, and AhbC. This phylogenetic occurrence profile strongly indicates that coproheme decarboxylase AhbD, discovered originally as part of the siroheme-to-protoheme pathway (14, 16), can also function in the HemQ-based coproporphyrin-dependent heme biosynthesis route described in this work. Given that AhbD is an anaerobic, radical SAM enzyme and that HemQ functions in the presence of oxygen, the presence of both coproheme decarboxylases in a single organism mimics the presence of an anaerobic coproporphyrinogen decarboxylase (HemN) and aerobic (HemF) coproporphyrinogen decarboxylase in many Gram-negative bacteria.

HemY most frequently coexists with HemQ and AhbD (where it would function as a coproporphyrinogen oxidase), but minimally with HemF and HemN (where it would function as a protoporphyrinogen oxidase) (Fig. 4). This strongly suggests that the annotation for HemY should be coproporphyrinogen oxidase, secondarily protoporphyrinogen oxidase. This finding is consistent with previous data showing that HemJ is the most common form of protoporphyrinogen oxidase in Proteobacteria (12, 41), whereas HemY is found infrequently among Proteobacteria that possess the classic pathway (Dataset S1). Attempts to delineate HemYs into coproporphyrinogen vs. protoporphyrinogen oxidases based upon sequence alignments proved futile (Fig. S6). Unlike ferrochelatases, where a clear sequence/structural distinction can be drawn due to the presence/absence of one active site lip, sufficient differences/commonality could not be found among available sequence data for HemYs. Additionally, no studies exist that characterize the range of substrate specificity (i.e., coproporphyrinogen vs. protoporphyrinogen) for HemYs to address the possibility that some HemYs may function in vivo with both substrates.

Discussion

The first significant study of porphyrin metabolism in Gram-positive bacteria was published in 1957 by Townsley and Neilands (20), who examined iron and porphyrin metabolism in the Gram-positive bacterium *Micrococcus lysodeikticus*. They reported that lysed cell preparations were capable of synthesizing coproheme, but not protoheme, and that no protoporphyrin was detectable. In the 1970s, the Jacobs' group (19) examined the terminal steps of heme synthesis in a variety of bacteria. They found that although the Proteobacteria *E. coli* and *Pseudomonas denitrificans* readily accumulated protoporphyrin when coproporphyrinogen was added to cell-free extracts, the Gram-positive bacteria *M. lysodeikticus* and *S. aureus* did not (19). Additionally, although *E. coli* and *P. denitrificans* accumulated protoporphyrin when the heme precursor ALA was provided, the Gram-positive bacteria accumulated



	Frequency of co-occurrence with:				% as only decarboxylase	Occurs with HemY (%)	Total genomes in selection
	HemF (%)	HemN (%)	HemQ (%)	AhbD (%)			
HemF	X	76.1	0.8	0.6	22.5	11	386
HemN	76.2	X	1.3	0.5	22.0	15	393
HemQ	2.0	3.3	X	27.6	67.1	93	152
AhbD	3.0	3.0	62.7	X	31.3	94	67

Fig. 4. Co-occurrence of HemF, HemN, HemQ, AhbD, and HemY. A Venn diagram illustrates the overlap of the four decarboxylases and HemY. The values shown in the table represent the percentage of each of the four decarboxylases as it is found to occur within the same genome with any of the other carboxylases. Also listed are the occurrence of each decarboxylase with HemY and the total number of genomes in the selection containing each decarboxylase. It should be noted that all heme-synthesizing Gram-positive bacteria possess HemY but few Gram-negative (Proteobacteria) bacteria have a *hemY* gene.

coproporphyrin and not protoporphyrin. Interestingly, neither group interpreted their observations as the basis for coproheme as a protoheme intermediate in Gram-positive bacteria but, instead, explained the lack of protoporphyrin and the ability to synthesize coproheme as interesting anomalies or artifacts of their in vitro systems.

We have demonstrated that within the Firmicutes, Actinobacteria, and evolutionary early-branching diderm taxa (39, 40), the terminal steps for the synthesis of protoheme do not go through protoporphyrinogen and protoporphyrin; instead, HemY oxidizes coproporphyrinogen to coproporphyrin, HemH inserts iron to form coproheme, and HemQ then decarboxylates the coproheme to form protoheme (Figs. 1 and 3). The manner in which Gram-positive HemY and HemH function should be highly similar to the action of the canonical HemY and HemH, respectively, because they differ only in that they use coproporphyrinogen and coproporphyrin, rather than protoporphyrinogen and protoporphyrin, as substrates. However, the mechanism by which HemQ functions is unclear at the present time. HemF and HemN, which catalyze the oxidative decarboxylation of coproporphyrinogen to protoporphyrinogen, function in a stepwise fashion with the production of monovinyl, monopropionyl porphyrinogen, followed by decarboxylation of a second ring propionate to make protoporphyrinogen and the overall production of two molecules of CO_2. HemF uses molecular oxygen as an electron acceptor, and the enzyme from *E. coli* has been shown to be stimulated by Mn (42). A model for catalysis by the HemF-type coproporphyrinogen oxidase proposed by Lash (43) has current acceptance. For the oxygen-independent HemN, Jahn and coworkers (2, 7) have presented evidence that this radical SAM enzyme forms a substrate radical by hydrogen abstraction at the β-carbon of the propionate side chain as the first step in the reaction sequence. For the iron-sulfur cluster containing the radical SAM enzyme AhbD, it has been proposed that it uses the same reaction mechanism as HemN, which may be reasonable, given that both are radical SAM enzymes that function in the absence of oxygen (14, 16).

HemQ differs from both HemF and HemN in that its substrate has a fully unsaturated macrocyclic tetrapyrrole with a coordinated iron. HemQ uses iron-coproporphyrin as a substrate, and the conversion by HemQ of coproheme to protoheme occurs for Fe-coproporphyrin but not for Ni-coproporphyrin, suggesting a possible role for iron in the reaction. The fate of the abstracted electrons appears not to be random but to involve specific acceptors. In vitro assays demonstrate that these acceptors may include free FMN or hydrogen peroxide, but not molecular oxygen, FAD, NAD, or NADP. Utilization of hydrogen peroxide is an attractive possibility because the antepenultimate enzyme HemY generates six molecules of peroxide that would provide for more than the four required for HemQ. Having multiple possible acceptors would provide for the metabolic diversity that many of the HemQ-containing bacteria possess.

A few oddities exist that do not clearly fit into either the classic protoporphyrin-dependent alternative (AhbA-, AhbB-, AhbC-, or AhbD-based) or coproporphyrin-dependent (HemQ- or AhbD-based) pathway. Such organisms are flagged in the associated SEED subsystem (23) with the variant codes "hybrid-1" or "hybrid-2" and are beyond the scope of this study. One group of note, however, is the photosynthetic organisms that produce heme via the coproporphyrin-dependent pathway. How they synthesize chlorophyll remains a mystery, given that all known chlorophyll-synthesizing pathways use protoporphyrin as an intermediate. Such cases are exceedingly rare: Of 72 photosynthetic organisms included in this study (23) (Dataset S1), only three species from two major taxonomic groups fall into this category, namely, *Heliobacterium modesticaldum* of the Gram-positive photosynthetic heliobacteria (44, 45) and *Roseiflexus* sp. RS-1 and *Roseiflexus castenholzi* of green nonsulfur bacteria (46, 47). These species are analyzed in more detail in *SI Methods*.

The inability of Firmicutes, Actinobacteria, and some evolutionarily early-branching diderm bacteria (39, 40) to synthesize protoporphyrin raises something of an evolutionary conundrum. If the general assumption is that aerobic metabolism arose after photosynthetic organisms provided atmospheric oxygen, how does one account for the presence of robust oxygen-using pathways among the Gram-positive bacteria whose tetrapyrrole biosynthetic pathway seems to predate the advent of photosynthesis?

The demonstration that Gram-positive bacteria use a different heme biosynthetic pathway that is specific for them, along with the observation that KO of *hemQ* in *S. aureus* results in small colony variants (18), suggests that this enzyme may represent a valid selective antimicrobial target for pathogenic Gram-positive bacteria. This finding could be of considerable value, given the advent of drug-resistant strains of *Staphylococcus*, *Listeria*, and *Mycobacteria* species. Antimicrobial resistance in Gram-positive pathogens has been recognized as a public health threat by both the WHO (48) and the US Centers for Disease Control and

Prevention (49). There are also identified links between the abundance of Firmicutes in the human gut microbiome and obesity and potentially cancer (50). HemQ, identified herein, is an essential enzyme for heme synthesis specific for Gram-positive bacteria capable of heme biosynthesis and is a novel pharmacological target with significant therapeutic relevance.

Methods

Expression, Purification, and Assay of HemY, HemH, and HemQ. Many of these procedures are previously published, and specific details are included in *SI Methods*.

Ultra Performance Liquid Chromatography Chromatograms of Reaction Products of HemY/H/Q Reactions. Analysis of products was performed by standard ultra performance liquid chromatography porphyrin/hemin analysis. Specific details are described in *SI Methods*.

Genomic Analysis. Many of these analyses are described in the text and/or in the Dataset S1. Additional details are presented in *SI Methods*.

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