A sterol reductase responsible for the unusual 8(14)-unsaturation in bacterial sterol production and degradation

Alysha K. Lee[a], José-Luis Giner[b], and Paula V. Welander[a]#

[a]Department of Earth Systems Science, Stanford University, Stanford CA 94305

[b]Department of Chemistry, State University of New York–Environmental Science and Forestry, Syracuse, NY 13210

#Address correspondence to Paula V. Welander, welander@stanford.edu

Abstract (248/250)

Sterols are a class of lipids that play a crucial role in human health through their essential physiological roles and as a point of interaction between commensal and pathogenic bacteria. The biosynthesis and modification of these lipids is a well-characterized process in many eukaryotes and increasingly in bacteria. However, the proteins responsible for formation of the unusual 8(14)-unsaturation found in the sterols produced by aerobic methanotrophs, dinoflagellates, nematodes, and marine sponges, remains unknown. Here, we utilize a heterologous expression system to identify a bacterial 8,14-sterol reductase (8,14-Bsr) responsible for generating the 8(14)-unsaturation in the aerobic methanotroph *Methylococcus capsulatus*. This enzyme modifies the direct product of C-14 demethylation, reducing one double bond in the nuclear core structure and isomerizing the other to produce an 8(14)-sterol. We subsequently tested the requirement of putative active site residues for catalysis through site directed mutagenesis, identifying residues likely involved in interacting with the sterol substrate and directly catalyzing this reaction. Bioinformatic analysis of the distribution of 8,14-Bsr reveals it is unique to the bacterial domain, found primarily in the *Methylococcaceae* family, the Mycobacteriales order, and yet uncultured members of the Myxococcota phylum. Further phylogenetic analysis of 8,14-Bsr suggests it shares an evolutionary history with the C-14 demethylase in these organisms and that these two enzymes were likely inherited together. These results provide insight into novel sterol biochemistry, further delimiting sterol biosynthesis in the bacterial domain from eukaryotes and illustrating the importance of molecular characterization to identify bacterial proteins that interact with sterols.

Importance (120/120)

Many of the eukaryotic proteins required for the biosynthesis of sterols, such as cholesterol, have been characterized. However, the pathways governing analogous processes in the bacterial domain are less characterized. Here, we identify an 8,14-sterol reductase in aerobic methanotrophs. This enzyme carries out a unique biochemical reaction, saturating and isomerizing double bonds in the nuclear core structure to produce an 8(14)-sterol. This reductase is restricted to the bacterial domain, further separating the evolution of bacterial sterol production from eukaryotes. Additionally, we find this reductase is prevalent in members of the sterol degrading order Mycobacteriales, highlighting a potential role for this protein in the remodeling of host sterol production by these pathogens.

Introduction

Sterols, such as cholesterol, have long been characterized in a eukaryotic context, with a rich body of literature describing the biosynthesis, regulation, and physiology of these lipids in a variety of eukaryotes (1–5). Sterols are also produced and modified sporadically throughout the bacterial domain (6–9). However, much less is known about the biology of these lipids in bacteria compared to their eukaryotic counterparts. Among bacterial sterol synthesizers, aerobic methanotrophs from the family *Methylococcaceae* have proved a tractable system from which to explore bacterial sterol production. Studies focused on these bacteria have revealed novel proteins involved in both the biosynthesis and trafficking of these lipids, setting bacterial sterol production apart from analogous processes in eukaryotes (10, 11). Such discoveries have challenged assumptions about the eukaryotic origins of sterol biosynthesis in bacteria and encouraged a more nuanced discussion about the early evolution of this ancient biosynthetic pathway. Yet there are

52 several steps in bacterial sterol biosynthesis that remain unexplored and characterization of these
53 missing biosynthetic steps could provide further insight into the evolution of sterol biosynthesis.

54 The sterols produced by *Methylococcaceae* are characterized by an unusual 8(14)-
55 unsaturation in the core ring structure (6, 12). The distinctive structure of these sterols, alongside
56 their unique isotopic signature derived from methanotrophy, has allowed these lipids to serve as
57 biomarkers, or molecular fossils, for aerobic methanotrophy in both modern and ancient
58 environments (13–15). Sterols with an 8(14)-unsaturation can also be found in other members of
59 both the eukaryotic and bacterial domains. Lipids with this modification are the primary sterol
60 produced by toxic dinoflagellate species belonging to the family *Kareniaceae* (16–18). The
61 biophysical properties of these lipids have been proposed as a key mechanism for immunity to the
62 toxins produced by *Kareniaceae* in both the dinoflagellates themselves and parasites specific to
63 these organisms (19). 8(14)-Unsaturations also characterize the hydroxylated sterols found in
64 sponges, corals, fungi, and a myxobacterium (20–24). While the capacity to produce an 8(14)-
65 unsaturation occurs in both eukaryotes and bacteria, the proteins responsible for carrying out this
66 step have yet to be characterized in either domain. Additionally, most aerobic methanotrophs lack
67 the canonical C-14 reductase to saturate the Δ14 double bond produced by C-14 demethylation. It
68 remains unclear if the Δ14 double bond in these bacteria is saturated in a distinct step from
69 production of the 8(14)-unsaturation, as is seen in eukaryotic sterol biosynthesis pathways, or if
70 these two steps happen simultaneously.

71 In this study, we use a heterologous expression system to identify a reductase responsible
72 for generating the 8(14)-unsaturation in aerobic methanotrophs. We find that this enzyme takes the
73 product of C-14 demethylation, which has two double bonds in the core ring structure, and both
74 saturates and isomerizes these double bonds to produce an 8(14)-sterol. Through site directed
75 mutagenesis, we then test the role of conserved active site residues, identifying several residues

76 essential for catalysis, and begin to consider the potential mechanism underlying this reaction. We
77 explore the taxonomic distribution of this protein and find this reductase is restricted to the
78 bacterial domain, occurring in other aerobic methanotrophs, uncultured myxobacteria, and
79 members of Mycobacteriales, including the pathogen *Mycobacterium tuberculosis*. Through our
80 phylogenetic analysis we reveal a close evolutionary relationship between this reductase and the
81 C-14 demethylase (Cyp51), which directly precedes it in sterol biosynthesis. Altogether, our
82 findings assign a biochemical function to a previously uncharacterized hypothetical protein found
83 both in environmental sterol producing and pathogenic sterol degrading bacteria while continuing
84 to differentiate bacterial sterol biology from similar processes in eukaryotes.

85 **Results**

86 **Identification of a bacterial 8,14-sterol reductase**

87 During sterol biosynthesis aerobic methanotrophs, like eukaryotes, demethylate sterols at
88 C-14 via the C-14 demethylase (Cyp51), resulting in a Δ8,14 unsaturation (25). This product is then
89 modified to produce the 8(14)-unsaturation characteristic of the sterols synthesized by this family.
90 However, apart from *Methylomicrobium* species (26), these methanotrophs do not harbor a
91 homolog to the canonical C-14 sterol reductase required to form the Δ8 unsaturation common in
92 eukaryotes, and the enzyme responsible for generating the 8(14)-unsaturation remains unknown
93 (Figure 1). To identify potential candidates for these modifications in aerobic methanotrophs, we
94 used a comparative genomics approach. We searched for genes in *Methylococcus capsulatus* that
95 are also present in *Methylobacter luteus, Methylomonas lenta*, and *Methyloprofundus sedimenti,*
96 members of *Methylococcaceae* that produce sterols, and are absent in the genomes of *Crenothrix*
97 *polyspora*, *Methylohalobius crimeensis*, and *Methylomonas methanica,* which belong to the same
98 order but do not produce sterols. This approach generated a list of 10 gene candidates (Table S1).

99 From this list, we identified a hypothetical protein that co-localizes with known sterol biosynthesis
100 genes in the genomes of several methanotrophs (Figure 1). This protein does not have sequence
101 homology to any characterized protein but belongs to the nuclear transport factor 2 (NTF2)
102 superfamily, which includes other proteins involved in sterol modification (27). Additionally, this
103 gene is present in the genomes of all sterol-producing aerobic methanotrophs. Given its wide
104 distribution and its co-localization with known sterol biosynthesis genes, we hypothesized that this
105 protein may be involved in performing either the reduction of the Δ14 unsaturation or generation of
106 the 8(14)-unsaturation.

107 We initially attempted to delete this gene (locus tag: H156DRAFT_MCAT2205) in
108 *Methylococcus capsulatus* through both an unmarked and marked approach, however neither of
109 these strategies were successful (Figure S1). Instead, to test the biochemical function of this
110 protein, we used a heterologous expression system that leverages a series of compatible plasmids
111 to produce the sterol biosynthesis intermediate lanosterol in *Escherichia coli*, which does not
112 produce sterols (28). Expression of the candidate protein MCAT2205 from *M. capsulatus* with
113 lanosterol as a substrate in *E. coli* resulted in no detectable activity, suggesting lanosterol may not
114 be a suitable substrate for this protein. To expand the potential substrates produced by our
115 heterologous expression system, we then introduced the fused C-14 demethylase-ferredoxin
116 homolog (Cyp51) from *M. capsulatus* (locus tag: H156DRAFT_MCAT1746). Expression of the C-14
117 demethylase resulted in production of 4,4-dimethylcholesta-8,14,24-trienol, a C-14 demethylated
118 sterol with an unsaturation at both Δ14 and Δ8. We then co-expressed the candidate MCAT2205
119 protein with the C-14 demethylase, and this resulted in complete conversion of all detectable 4,4-
120 dimethylcholesta-8,14,24-trienol to 4,4-dimethylcholesta-8(14), 24-dienol, a product of sterol
121 biosynthesis in aerobic methanotrophs (Figure 2; Figure S2) (29). We further confirmed the
122 production of the dienol sterol by NMR analysis (data not shown). These results demonstrate that

123 this enzyme is a bacterial 8,14-sterol reductase (8,14-Bsr), and it is sufficient to perform both a
124 reduction and isomerization reaction to remove the Δ14 and Δ8 double bonds, generating the 8(14)-
125 double bond typically observed in the sterols produced by aerobic methanotrophs.

126 **Site directed mutagenesis of conserved residues does not separate the reduction and**
127 **isomerization functions of 8,14-Bsr.**

128 Sterol biosynthesis is characterized by examples of bifunctional enzymes, processive
129 reactions, and allylic mechanisms (30–33). We were curious if we could provide further insight into
130 the potential mechanism of 8,14-Bsr, which appears to reduce one double bond in the ring
131 structure and isomerize the other, through site directed mutagenesis of potential active site
132 residues. We hypothesized that if the reaction performed by 8,14-Bsr involves a distinct reduction
133 reaction followed by the isomerization of the remaining double bond, we might be able to
134 mutagenize the active site to result only in the production of a reduced intermediate. To identify
135 potential amino acid residues involved in catalysis, we first compared a structural model of 8,14-
136 Bsr to the structure of ketosteroid isomerase (PBD#1OH0), a well characterized NTF2 family protein
137 that functions on steroids (27, 34). Ketosteroid isomerase (Ksi) harbors a hydrophobic active site
138 pocket found between the alpha helices B and C and catalysis by Ksi requires several tyrosine and
139 aspartic acid residues found within this pocket (35). A structural model of 8,14-Bsr shares the
140 same cone shaped fold as other NTF2 family proteins and, like ketosteroid isomerase, has a
141 hydrophobic pocket between helices B and C. However, the active site residues found in Ksi do not
142 align with those in 8,14-Bsr. Instead, we identified two aspartic acids (D133 and D136) and four
143 tyrosine residues (Y37, Y43, Y78 and Y80) present in this pocket that are largely conserved among
144 other homologs of this protein (Figure S3).

145 To test if these residues are involved in catalysis, we first individually mutated each aspartic
146 acid residue to alanine and co-expressed these variants with the C-14 demethylase in our *E. coli*
147 heterologous expression system. Mutating the aspartic acid residue at position 133 to alanine
148 (D133A) resulted in the accumulation of the substrate 4,4-dimethylcholesta-8,14,24-trienol,
149 indicating a decrease in 8,14-Bsr function and suggesting this residue is involved in the enzymes
150 mechanism. Conversely, mutating the aspartic acid at position 136 to alanine (D136A) had no
151 effect on enzyme function (Figure 3a). We next individually mutated each tyrosine residue to either
152 phenylalanine, removing the hydroxyl group but maintaining the aromaticity of the side chain, or
153 alanine, removing both the hydroxyl group and aromaticity of the side chain, and co-expressed
154 these variants as well. Mutating the tyrosine at position 80 to either phenylalanine (Y80F) or alanine
155 (Y80A) had no effect on the enzyme function in this system (Figure 3b). The Y37F and Y78F
156 mutations also had no effect on enzyme function. However, mutating these residues to alanine
157 (Y37A or Y78A) resulted in reduced enzyme function, suggesting the aromaticity of these two
158 tyrosine residues plays an important role in performing this reaction, perhaps through pi-stacking
159 interactions with the rings of the sterol substrate (Figure 3c). A broader search of 8,14-Bsr homologs
160 identifies one in the Actinobacterium *Haloenchinothrix aidingensis* with a phenylalanine residue at
161 position 37, illustrating a biologically relevant example of this mutation. Finally, mutating Y43 to
162 either phenylalanine (Y43F) or alanine (Y43A) resulted in decreased enzyme function, suggesting
163 the hydroxyl group of this tyrosine residue may be directly involved in catalysis (Figure 3d).

164 While we were able to identify residues in the active site pocket important for the reaction
165 carried out by 8,14-Bsr, we were unable to generate a mutant which only performs the reduction
166 reaction suggesting that these two steps may be carried out through a single reaction mechanism.
167 This could occur through allylic rearrangement after the reduction of the Δ14 double bond. The
168 canonical Δ14 reductase saturates the Δ14 double by the addition of a hydride from NADPH at C-15

169 followed by protonation at C-14 to generate a Δ8 sterol (36). We propose that 8,14-Bsr, like the
170 canonical Δ14 reductase, reduces its substrate by hydride addition at C-15. It is possible this
171 enzyme also uses NADPH as a hydride donor during this process, however 8,14-Bsr has no
172 sequence homology to known cofactor binding sites. Hydride addition is then followed by
173 protonation of an allylic anion intermediate at C-9 to generate an unsaturation at 8(14) (Figure S4).
174 Additional biochemical and structural characterization of this enzyme is needed to confirm this
175 mechanism, identify the role of potential cofactors, and determine the function of specific residues
176 in catalyzing this unique reaction.

177 **8,14-Bsr is a sterol biosynthesis protein restricted to the bacterial domain**

178 Sterols with an 8(14)-unsaturation are found in a diverse array of bacteria and eukaryotes.
179 To identify if 8,14-Bsr is responsible for 8(14)-double bond formation in these other organisms, we
180 conducted BLASTp and tBLASTn searches against the genomes and transcriptomes present in the
181 IMG and NCBI databases (e-value cutoff e-05). Among eukaryotes, including those that produce
182 8(14)-sterols such as dinoflagellates and nematodes (37, 38), we were unable to identify any
183 homologs to the bacterial 8,14-sterol reductase. Furthermore, many of the eukaryotes that produce
184 8(14)-sterols harbor homologs to the C-14 reductase (Table S2), suggesting that either these C-14
185 reductase homologs protonate sterols at C-9 during catalysis, like 8,14-Bsr, or that these
186 eukaryotes require an additional, unidentified enzyme to generate the 8(14)-unsaturation. In either
187 case, the biosynthesis of 8(14)-sterols provides an additional instance of independent evolution
188 between sterol production in the eukaryotic and bacterial domains.

189 While 8,14-Bsr is absent from the eukaryotic domain, homologs are present in a small but
190 diverse group of bacteria. This includes all sterol-producing aerobic methanotrophs, even
191 *Methylomicrobium* species which also harbor a C-14 reductase. 8,14-Bsr homologs from

192 *Methylococcaceae* cluster together and broadly recapitulate 16S rRNA phylogeny (Figure S5).
193 These phylogenetic patterns, alongside the wide distribution of the bacterial 8,14-sterol reductase
194 in aerobic methanotrophs, suggests that the 8(14)-double bond is an ancestral feature of sterol
195 biosynthesis in the family *Methylococcaceae*. 8,14-Bsr homologs are also found in the genomes of
196 γ-proteobacteria, α-proteobacteria, Mycobacteriales, and uncultured myxobacteria. Several of
197 these bacteria are characterized sterol degraders and consume sterols acquired from the
198 environment as a carbon source (39–42). To confirm these homologs can function as 8(14)-sterol
199 reductases, we co-expressed homologs from the uncultured myxobacteria *Sandaracinus*
200 *amylolyticus* NAT-131 and the sterol degrader *Mycobacterium tuberculosis* in our *E. coli*
201 heterologous system (Figure 4a). In both cases, these homologs produced the same 8(14)-sterols
202 we observe from heterologous expression of the *M. capsulatus* homolog, suggesting the capacity to
203 synthesize 8(14)-sterols, either de novo or from exogenously acquired sterol precursors, is present
204 in these disparate phyla as well.

205 Across these bacteria, 8,14-Bsr often co-localizes with the C-14 demethylase (Figure 4b).
206 Phylogenetic analysis of the C-14 demethylases that co-occur with 8,14-Bsr reveals that these C-
207 14 demethylase homologs form a distinct clade from C-14 demethylases found in other bacteria
208 and eukaryotes, even in the case of sequences from Mycobacteriales and *Streptomyces* despite
209 both belonging to the phylum Actinomycetes (Figure 4c; Figure S6-S7). Additionally, these C-14
210 demethylase homologs are all fused or co-localized with their cognate ferredoxin (26), which is not
211 observed of other C-14 demethylases (Figure S8). Broadly, the branching pattern of this clade of C-
212 14 demethylases largely mirrors what we observe from 8,14-Bsr, suggesting these two enzymes
213 have co-evolved in the sterol-producing and sterol-degrading bacteria that harbor this distinct
214 protein pair.

215 **Discussion**

216 Here, we identified a sterol reductase that generates an unsaturation at 8(14) through the
217 reduction and allylic rearrangement of the Δ14 and Δ8 double bonds to result in the unusual
218 nuclear core structure configuration that characterizes sterols in aerobic methanotrophs. The
219 bacterial 8,14-sterol reductase shares no sequence homology to any proteins with known
220 functions, illustrating the importance of coupling molecular characterization to bioinformatic
221 analysis when identifying bacterial proteins involved in sterol biosynthesis, as bioinformatic
222 analyses alone were not sufficient to predict the function of this enzyme. While many proteins and
223 motifs that are diagnostic for sterol binding have been identified in eukaryotes, they are largely
224 absent in bacterial sterol producers. Instead, sterol-producing bacteria continue to present an
225 opportunity to uncover novel sterol biochemistry, better informing how we consider the biological
226 and evolutionary relationships governing sterol modification across the bacterial domain. This has
227 been particularly true in aerobic methanotrophs, which have been previously shown to use unique
228 proteins in both the synthesis and transport of sterols (10, 11). By continuing to characterize
229 bacterial sterol biosynthesis and physiology, we will gain a more complete picture of the
230 biochemistry surrounding these important lipids.

231 Our work also illustrates the biotechnological utility in developing heterologous systems to
232 study complex biosynthesis pathways. Expression of the fused C-14 demethylase-ferredoxin
233 homolog from *M. capsulatus* resulted in production of the fertility hormone follicular fluid-meiosis
234 activating sterol (FF-MAS). FF-MAS is found in high abundance in mammalian follicular fluid where
235 it is involved in regulation of oocyte maturation (43, 44) and this accumulation is important in the
236 resumption of meiosis in human, mouse, and porcine oocytes during ovulation (45–47). While FF-
237 MAS has proved promising in increasing successful culturing of porcine and mouse oocytes in vitro

238 (47–50), it remains unclear if there may be a role for FF-MAS in the culturing of human oocytes for in
239 vitro fertilization treatments (51, 52). Complicating the study and use of this hormone in a clinical
240 setting, FF-MAS is not present in high concentrations from extractable sources. Currently, a 9-step
241 chemical synthesis scheme for FF-MAS has been developed for production (53). Heterologous
242 expression may present an alternative and more sustainable pathway for generating FF-MAS and,
243 with further optimization, prove a useful source of this lipid for both clinical and research purposes.

244 Here, we also show that 8,14-Bsr is entirely absent from the eukaryotic domain. The
245 absence of this protein from eukaryotic genomes, particularly those of the dinoflagellates,
246 sponges, and nematodes that produce 8(14)-unsaturated sterols, points to convergent evolution
247 underlying this modification in the two domains. However, how eukaryotes generate 8(14)-sterols
248 remains an open question. This reaction could conceivably be catalyzed by an ortholog to the
249 canonical C-14 reductase that protonates at the C-9 position, as we have proposed for 8,14-Bsr, to
250 generate an 8(14)-sterol. Alternatively, generation of the 8(14)-unsaturation could also be catalyzed
251 by an isomerase after the canonical C-14 reductase produces a Δ8 sterol. The treatment of *Karenia*
252 *brevis*, a toxic dinoflagellate that synthesizes 8(14)-sterols, with the fungicide fenpropidine, an
253 inhibitor of both the C-14 reductase and C-8 isomerase, resulted in accumulation of Δ8,14,22
254 sterols (16), suggesting an ortholog to one of these two enzymes is responsible for generating 8(14)-
255 sterols. Additionally, *C. elegans*, which modifies exogenously acquired sterols, has been
256 hypothesized to generate 8(14) sterols through an ortholog to the eukaryotic C-8 sterol isomerase
257 that would isomerize the Δ7 double bond to 8(14) (54). Further functional characterization of both
258 the C-14 reductase and C-8 isomerase orthologs in these organisms could resolve these
259 outstanding questions, better clarifying the evolutionary trajectory of 8(14)-sterol production in
260 eukaryotes.

261 The independent evolution of enzymes responsible for producing 8(14)-unsaturated sterols
262 in eukaryotes and bacteria also highlights the biological importance of these small modifications to
263 the biophysical properties and physiological functions of these lipids in the cell. In the case of
264 either domain, the physiological role of the 8(14)-unsaturation remains unknown. Sterols with an
265 8(14)-unsaturation from the toxic dinoflagellate *K. brevis* have a protective effect against the toxins
266 produced by this organism (19). These sterols have also been proposed as a potential deterrent for
267 predation as they might interfere with predator sterol metabolism (55). However, the sterols from
268 these dinoflagellate species are also characterized by unique modifications to the sterol side chain
269 (38) and the effect of these side chain modifications versus the nuclear core unsaturation on
270 membrane dynamics is unknown. Similarly, we do not know the physiological function of this
271 modification in sterol-producing bacteria, such as the aerobic methanotrophs. Yet it is interesting
272 to note that while 8,14-Bsr is present in all sterol producing aerobic methanotrophs,
273 *Methylomicrobium* species also harbor a C-14 reductase and C-8 isomerase suggesting these
274 bacteria are capable of producing sterols with different double bond configurations in the core
275 structure. This pathway bifurcation again highlights the potential importance of these small sterol
276 modifications in *Methylococcaceae* and suggests a possibility for specific functions tied to specific
277 sterols.

278 Finally, our confirmation of 8,14-Bsr activity in sterol-degrading bacteria also raises
279 questions about the function of these enzymes in Mycobacteriales. Pathogens from this family are
280 well known for their ability to catabolize host sterols, specifically cholesterol, and the enzymes
281 involved in this process are essential for pathogenicity (56). However, less is known about how
282 these bacteria interact with other sterols produced by the host. The C-14 demethylase from several
283 *Mycobacterium* species have been crystalized and characterized, demonstrating that these
284 enzymes are functional C-14 demethylases (57–59). While neither the C-14 demethylase nor 8,14-

285 Bsr are essential to cell viability or pathogenicity (60–62), the presence of these two genes in
286 *Mycobacterium* species would suggest these host-associated bacteria have the capacity to modify
287 exogenously acquired lanosterol. Indeed, a study found that when fed lanosterol as a carbon
288 source, a *Mycobacterium* sp. converted this sterol into a C_{19} steroid with an 8(14)-unsaturation in
289 the ring structure (63, 64). Mycobacteria include intracellular pathogens that persist in
290 macrophages. Macrophages accumulate lanosterol early in bacterial infection in response to Toll-
291 like receptor 4 activation, triggering downstream signaling pathways and increasing membrane
292 fluidity to allow for increased phagocytosis (65). Perhaps the C-14 demethylase and 8,14-Bsr are
293 used by *Mycobacterium* to remodel sterol production in these macrophages during infection. In
294 support of this, 8,14-Bsr was found to be upregulated in *M. tuberculosis* early in macrophage
295 infection (66, 67). Further research investigating the role of lanosterol degradation in
296 *Mycobacterium* physiology could yield additional insight into the pathogenic life cycle of these
297 bacteria. Altogether, our findings provide a foundation for probing new questions around sterol
298 physiology in both sterol producing and sterol degrading bacteria while highlighting the utility in
299 studying environmental bacteria, such as *Methylococcaceae*, to explore basic and fundamental
300 biological processes.

301 **Methods**

302 **Bacterial Culture.**

303 For genomic DNA extraction, *Methylococcus capsulatus* Texas was grown in liquid culture
304 on NMS media supplemented with methane for 3 days at 37°C (68). *Escherichia coli* DH10B was
305 used for all cloning and expression experiments and cultured on either Luria Broth (LB) or TYGPN
306 media. In heterologous expression experiments, *E. coli* DH10B was cultured in TYPGPN medium, at
307 an initial temperature of 37°C and cultures were shifted to 30°C at expression induction for 40

308 hours, shaking at 220 rpm. Cultures were supplemented with 100μg/ml of carbenicillin, 20μg/ml of
309 chloramphenicol, 30μg/ml of kanamycin, and/or 15μg/ml of gentamycin as needed

310 **Molecular Cloning.**

311 All oligonucleotides and plasmids used in this study are described in Table S3 and S4.
312 Oligonucleotide primers were ordered from Integrated DNA Technologies (Coralville, IA). Plasmids
313 were isolated from *E. coli* through the GeneJET Plasmid Miniprep Kit (Thermo Scientific). DNA
314 fragments used in cloning were gel purified through the GeneJET Gel Extraction Kit (Thermo
315 Scientific). Constructed plasmids were sequenced by ELIM Biopharm (Hayward, CA).

316 All plasmids were constructed using sequence and ligation independent cloning (SLIC) (69).
317 Briefly, gene fragments were amplified using Phusion DNA Polymerase (New England Biolabs) with
318 complementary plasmid insertion site overhangs and gel purified. The expression plasmids
319 pBAD1031K or pSRKGm-lacUV5-rbs5 were linearized by digestion using either NcoI or NdeI (New
320 England Biolabs), respectively. Complementary overhangs were created on both gel-purified gene
321 fragments and linearized plasmids by incubation with T4 DNA Polymerase (MilliporeSigma) without
322 nucleotides. Vectors and gene fragments were then annealed and transformed by electroporation
323 without ligation into *E. coli*.

324 **Site Directed Mutagenesis.**

325 Site directed mutagenesis was performed by PCR amplification of the vector with a single
326 oligonucleotide with the desired change. This reaction was performed using 2.5 units of PfuUltra II
327 Fusion HS DNA Polymerase (Agilent) with the oligonucleotide (0.2 μM), 0.2 mM dNTPs, and 50ng
328 plasmid DNA in 1x PfuUltra buffer and a 1-minute per kilobase pair extension time at 68 °C. The
329 reaction was then digested with DpnI (New England Biolabs) and transformed by electroporation

330 into *E. coli*. The mutation was verified through plasmid sequencing by ELIM Biopharm (Hayward,
331 CA).

332 **Heterologous Expression.**

333 Biosynthesis genes for production and cyclization of oxidosqualene were expressed from a
334 set of compatible plasmids in *E. coli* DH10B (29). Heterologous expression strains were
335 constructed as described in Table S5. Genes of interest were expressed from either the IPTG-
336 inducible plasmid pSRKGm-*lac*UV5-rbs5 or the arabinose-inducible plasmid pBAD1031K. Strains
337 were cultured in 20ml of TYPGN media supplemented with the necessary antibiotics, at 37°C,
338 shaking at 220 rpm, until mid-log phase. At mid-log phase, expression was induced with 500μM
339 IPTG and 0.2% (wt: vol) arabinose and cultures were shifted to 30°C, shaking at 220 rpm, for 30-40
340 hours before harvesting cultures by centrifugation at 4,500xg for 10 minutes.

341 **Lipid Extraction.**

342 Lipids were extracted through a modified Bligh-Dyer extraction (70). Cell pellets were
343 sonicated in a water bath in 10:5:4 methanol: dichloromethane (DCM): water (vol: vol: vol) for 1
344 hour. Lipids were then phase separated by addition of two times the volume 1:1 DCM: water (vol:
345 vol) and the organic layer removed and dried under nitrogen gas. Lipids were then derivatized to
346 either trimethylsilyl ethers by adding 1:1 (vol: vol) Bis(trimethylsilyl)trifluoroacetamide: pyridine and
347 heating at 70°C for 1 hour or derivatized to acetyl groups by adding 1:1 (vol: vol) acetic anhydride:
348 pyridine and heating at 70°C for 1 hour and resuspending in DCM.

349 **Gas Chromatography Mass Spectrometry (GC-MS).**

350 Sterols were separated on an Agilent 7890B Series GC using a 60 m Agilent DB17HT column
351 (60 m x 0.25 mm i.d. x 0.1 μm film thickness) with helium as the carrier gas at constant flow of

352 1.1 mL/min and programed as follows: 100°C for 2 minutes; then 8°C/minute to 250°C and held for
353 10 minutes; then 3°C/minutes to 330°C and held for 17 minutes. 2 µL of each sample was injected
354 in splitless mode at 250°C. The GC was coupled to a 5977 A Series MSD with the ion source held at
355 230°C and operated at 70 eV in EI mode scanning from 50-850 Da in 0.5 s. Lipids were analyzed
356 using Agilent MassHunter Qualitative Analysis (B.06.00) and identified based on retention time and
357 spectra by comparison to previously confirmed laboratory standards, spectra deposited in the
358 National Institute of Standards and Technology (NIST) databases or previously published spectra as
359 well as through NMR analysis.

360 **Bioinformatics.**

361 Homologs of the bacterial 8(14)-sterol reductase (locus tag: H156DRAFT_MCAT2205) and
362 the C-14 demethylase (locus tag: H156DRAFT_MCAT1746) were identified using a BLASTp search
363 (e-value cutoff of e-30) of the cultured genomes available in the JGI IMG and NCBI databases (71–
364 73). BLAST searches against eukaryotic genomes also included a tBLASTn search of the
365 transcriptome database in NCBI (e-value cutoff e-05). Protein alignments were performed in MAFFT
366 (version 7.526) using the L-INS-I algorithm with 1000 iterations (74, 75). Phylogenetic trees were
367 generated using IQ-TREE2 (version 3.0.1). Modelfinder was used to identify the best fit model for
368 both bacterial 8(14)-sterol reductase and the C-14 demethylase trees and a maximum likelihood
369 tree was generated with 5000 ultrafast bootstrap replicates (76, 77). Phylogenetic trees were then
370 edited for figures using the iTOL website (http://itol.embl.de/).

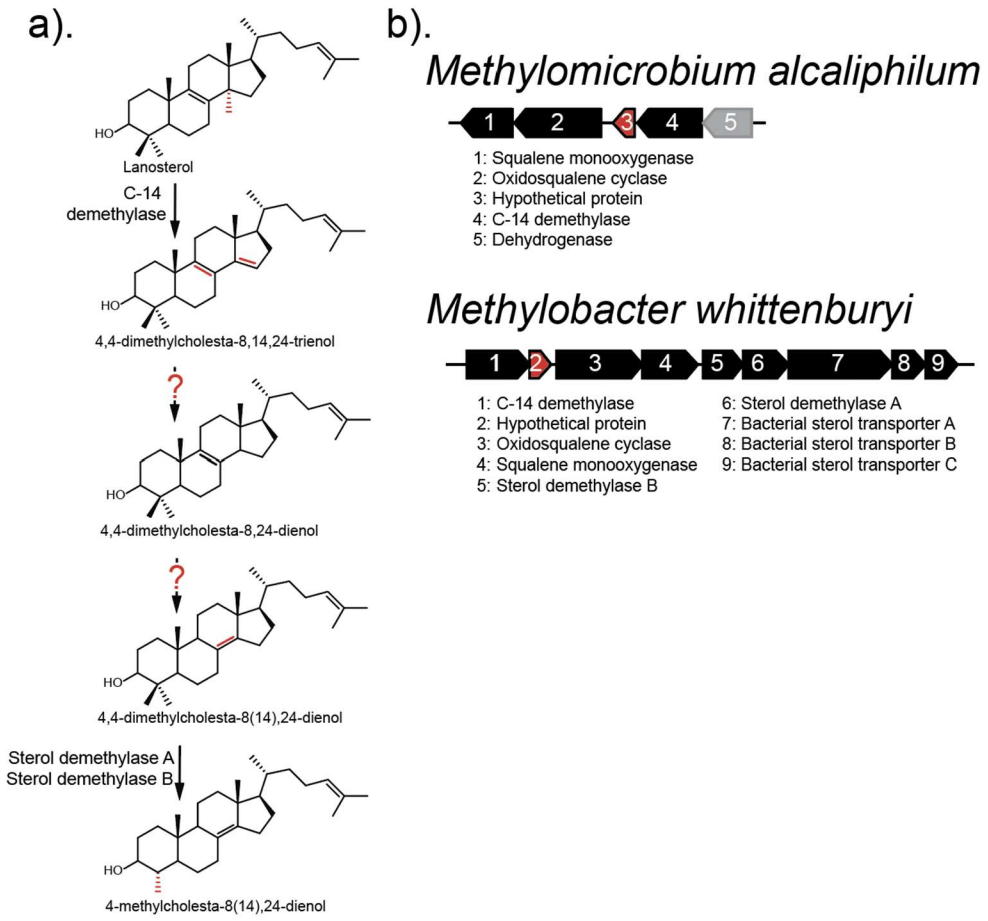
371 Models of bacterial 8,14-sterol reductase homologs were generated using ColabFold
372 alphafold2 platform (version 1.5.5)(78). Ketosteroid isomerase was identified as a structurally
373 similar protein based on structural similarity search through the RCSB Protein Database using the
374 alpha model of the *M. capsulatus* bacterial 8,14-sterol reductase homolog (79). Bacterial 8,14-

375 sterol reductase was aligned to the crystal structure of ketosteroid isomerase (PDB: 1OH0) in
376 Pymol (version 2.5.7) using the cealign function. The active site pocket of both proteins was
377 visualized using the set_surface_mode, 2 command and colored by hydrophobicity using the color
378 h script (80).

379 **Acknowledgements**

380 We thank the members of the Welander lab for their helpful discussions and suggestions. Portions
381 of the experimental work were performed in the Stanford Geomicrobiology Shared Laboratories
382 Core Facility (RRID:SCR_025000). Funding for this study was provided by National Science
383 Foundation Award 1919153 (to P.V.W).

384 **Figures**



385

386 **Figure 1. Aerobic methanotrophs produce 8(14)-unsaturated sterols by an unknown**
387 **mechanism.** a) The sterol biosynthesis pathway in aerobic methanotrophs. After cyclization of
388 oxidosqualene, the C-14 methyl group is removed from lanosterol by the C-14 demethylase
389 (Cyp51), generating a Δ14,15 double bond. It is unknown how the Δ14,15 double bond is removed,
390 or the 8(14)-double bond generated in these bacteria. In some methanotrophs, sterols are further
391 demethylated once at C-4 by the sterol demethylase AB (sdmAB) proteins. b) Examples of sterol
392 biosynthesis gene clusters in methanotrophs, featuring known sterol biosynthesis and transport
393 genes (black), a gene without a known function in sterol biosynthesis (grey), and the hypothetical
394 protein characterized in this study (red).

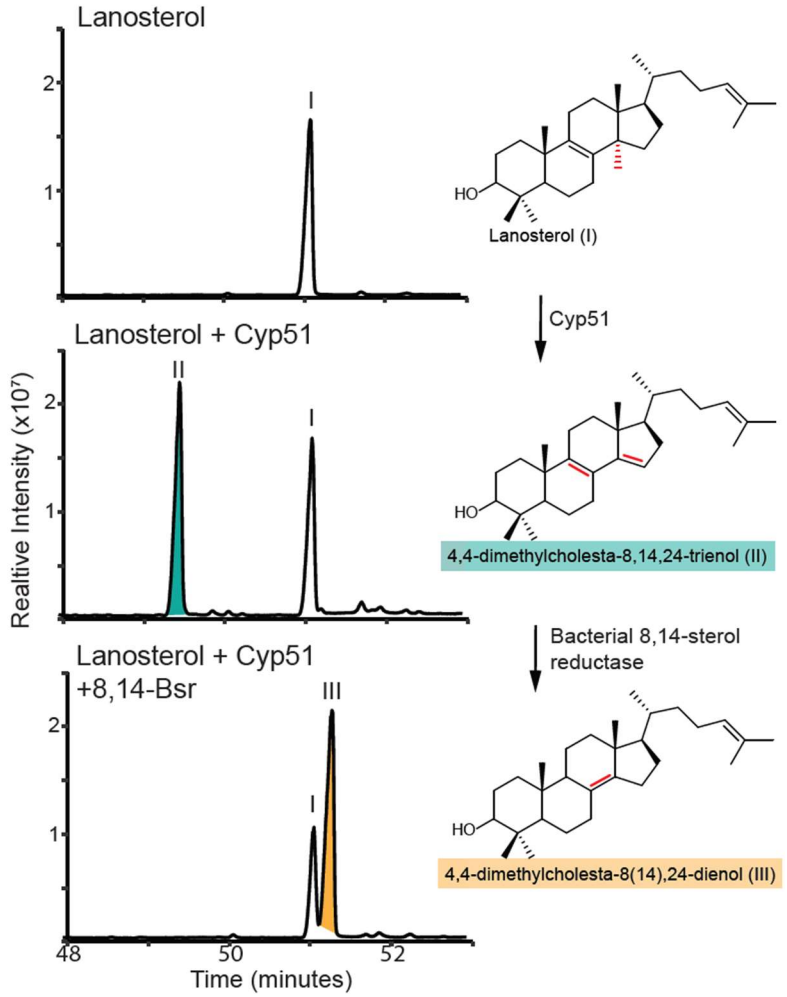


Figure 2. Bacterial 8,14-sterol reductase (8,14-Bsr) generates an 8(14)-unsaturation from 4,4-dimethylcholesta-8,14,24-trienol. Total ion chromatograms from heterologous *E. coli* expression cultures. Expression of the C-14 demethylase (Cyp51) in a lanosterol producing strain of *E. coli* results in production of 4,4-dimethylcholesta-8,14,24-trieneol (II; teal). Co-expression of 8,14-Bsr with Cyp51 results in production of 4,4-dimethylcholesta-8(14), 24-dienol (III; orange). All lipids were derivatized to trimethylsilyl ether groups. Mass spectra of identified sterols are shown in Figure S2.

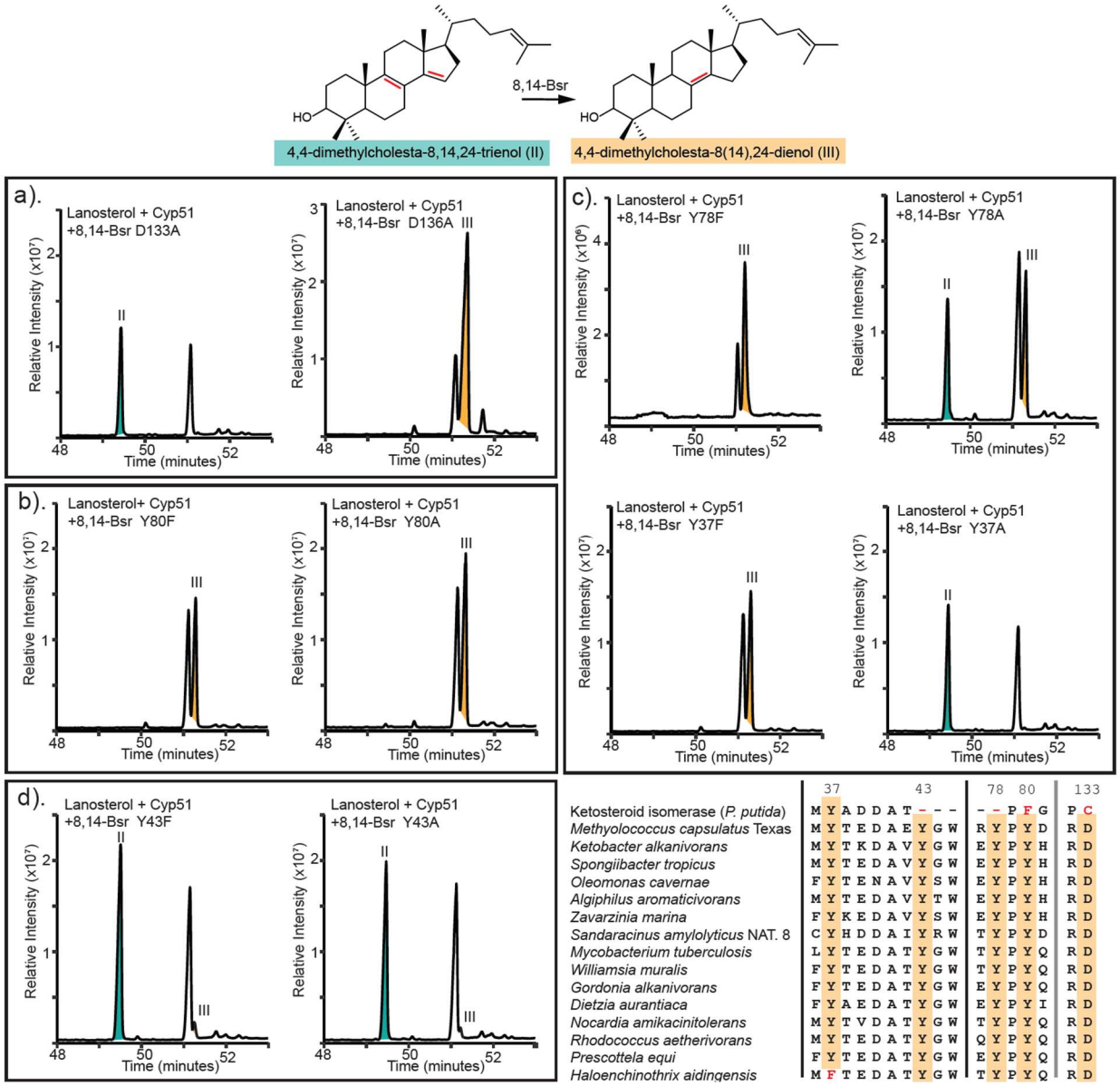


Figure 3. Site directed mutagenesis of conserved residues in 8,14-Bsr reveals residues essential for catalysis. Total ion chromatograms of lipids extracted from heterologous expression of Cyp51 with putative 8,14-Bsr active site pocket mutants. Peaks corresponding to the substrate for 8,14-Bsr, 4,4-dimethylcholesta-8,14,24-trienol, are labeled teal and the product, 4,4-dimethylcholesta-8(14)-dienol, are labeled orange. a) Mutation of the aspartic acid at position 133 and 136 to alanine. b) Mutations of the tyrosine at position 80 to either phenylalanine or alanine,

412 showing no impact on 8,14-Bsr function. c) Mutations of the tyrosine at position 78 and 37 to either
413 phenylalanine or alanine, where only mutations to alanine impact function. d) Mutations of the
414 tyrosine at position 43 to either phenylalanine or alanine, where both mutation to phenylalanine
415 and alanine impact function. An alignment of these conserved residues in 8,14-Bsr homologs is
416 included for reference.

417

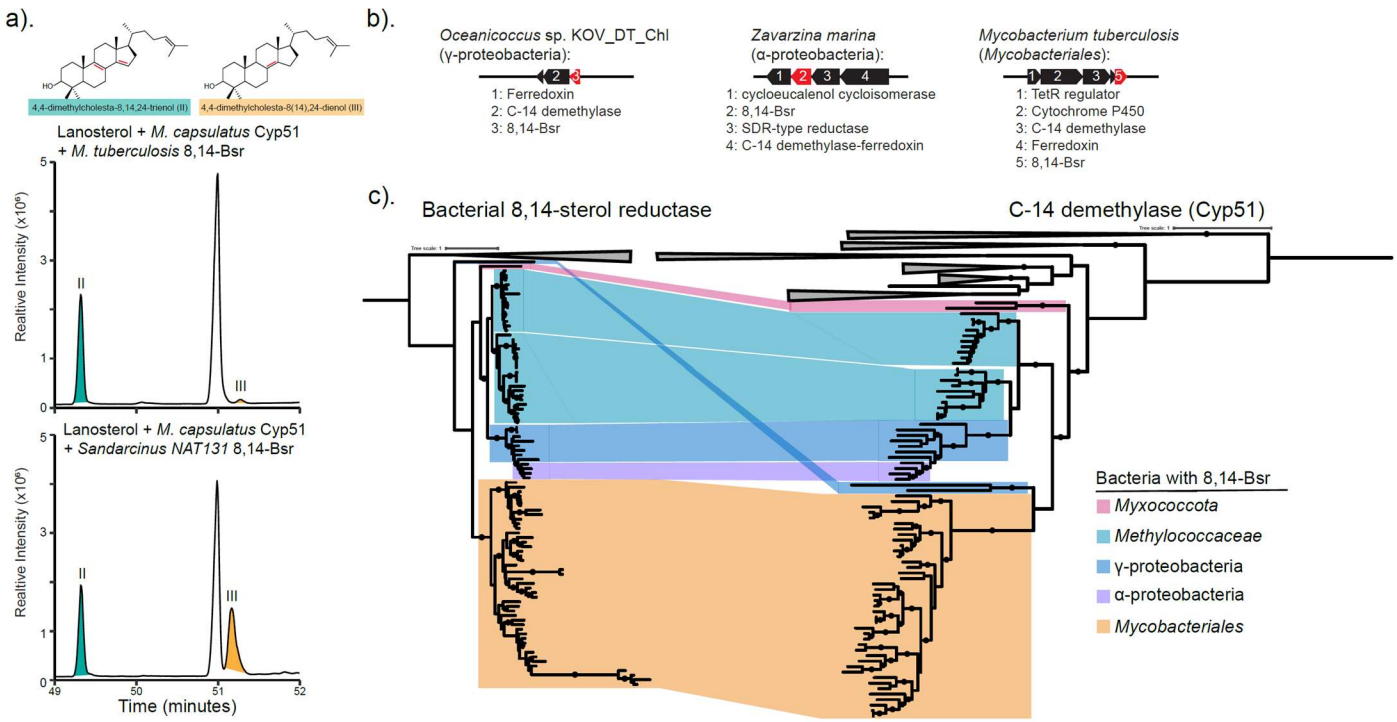


Figure 4. The bacterial 8,14-sterol reductase (8,14-Bsr) is found in diverse sterol producing and sterol degrading bacteria. a) Total ion chromatograms of lipids extracted from heterologous expression of Cyp51 from *Methylococcus capsulatus* Texas with either the 8,14-Bsr homolog from *Mycobacterium tuberculosis* or the uncultured myxobacteria *Sandaracinus* sp. NAT131. Peaks corresponding to the substrate for 8,14-Bsr, 4,4-dimethylcholesta-8,14,24-trienol, are labeled teal (II) and the product, 4,4-dimethylcholesta-8(14)-dienol, orange (III). b) Representative gene clusters from diverse bacteria where Cyp51, the Cyp51 ferredoxin, and 8,14-Bsr are co-localized. 8,14-Bsr is featured in red. c) Maximum likelihood phylogenetic trees of bacterial 8,14-sterol reductase (8,14-Bsr) with ketosteroid isomerase (Ksi) as an outgroup and Cyp51 with Cyp4 as an outgroup were generated using IQTree with the model of best fit and 5000 ultrafast bootstrap replicates. Branches with bootstrap support values >90 are denoted with a black circle. Sequences from different taxonomic groups which harbor 8,14-Bsr homologs are mapped onto the Cyp51 tree and colored by

their taxonomic group as follows: Myxococcota (pink), *Methylococcaceae* (Cyan), γ-proteobacteria (dark blue), α-proteobacteria (purple), and Mycobacteriales (orange).

References

1. Bloch K. 1965. The Biological Synthesis of Cholesterol. Science 150:19–28.

2. Róg T, Pasenkiewicz-Gierula M, Vattulainen I, Karttunen M. 2009. Ordering effects of cholesterol and its analogues. Biochimica et Biophysica Acta (BBA) - Biomembranes 1788:97–121.

3. Anderson RH, Sochacki KA, Vuppula H, Scott BL, Bailey EM, Schultz MM, Kerkvliet JG, Taraska JW, Hoppe AD, Francis KR. 2021. Sterols lower energetic barriers of membrane bending and fission necessary for efficient clathrin-mediated endocytosis. Cell Reports 37:110008.

4. Nes WD. 2011. Biosynthesis of Cholesterol and Other Sterols. Chem Rev 111:6423–6451.

5. Eberlé D, Hegarty B, Bossard P, Ferré P, Foufelle F. 2004. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 86:839–848.

6. Wei JH, Yin X, Welander PV. 2016. Sterol Synthesis in Diverse Bacteria. Front Microbiol 7:990.

7. Pearson A, Budin M, Brocks JJ. 2003. Phylogenetic and biochemical evidence for sterol synthesis in the bacterium Gemmata obscuriglobus. Proceedings of the National Academy of Sciences 100:15352–15357.

8. Kohl W, Gloe A, Reichenbach H. 1983. Steroids from the Myxobacterium Nannocystis exedens. Microbiology 129:1629–1635.

9. Bird CW, Lynch JM, Pirt FJ, Reid WW, Brooks CJW, Middleditch BS. 1971. Steroids and Squalene in Methylococcus capsulatus grown on Methane. Nature 230:473–474.

10. Lee AK, Banta AB, Wei JH, Kiemle DJ, Feng J, Giner J-L, Welander PV. 2018. C-4 sterol demethylation enzymes distinguish bacterial and eukaryotic sterol synthesis. Proceedings of the National Academy of Sciences 115:5884–5889.

11. Zhai L, Bonds AC, Smith CA, Oo H, Chou JC-C, Welander PV, Dassama LM. 2024. Novel sterol binding domains in bacteria. eLife 12:RP90696.

12. Schouten S, Bowman JP, Rijpstra WIC, Sinninghe Damsté JS. 2000. Sterols in a psychrophilic methanotroph, Methylosphaera hansonii. FEMS Microbiology Letters 186:193–195.

13. Bouloubassi I, Nabais E, Pancost RD, Lorre A, Taphanel M-H. 2009. First biomarker evidence for methane oxidation at cold seeps in the Southeast Atlantic (REGAB pockmark). Deep Sea Research Part II: Topical Studies in Oceanography 56:2239–2247.

14. Elvert M, Niemann H. 2008. Occurrence of unusual steroids and hopanoids derived from aerobic methanotrophs at an active marine mud volcano. Organic Geochemistry 39:167–177.

15. Coolen MJL, Hopmans EC, Rijpstra WIC, Muyzer G, Schouten S, Volkman JK, Sinninghe Damsté JS. 2004. Evolution of the methane cycle in Ace Lake (Antarctica) during the Holocene: response of methanogens and methanotrophs to environmental change. Organic Geochemistry 35:1151–1167.

16. Leblond JD, Roche SA, Porter NM, Howard JC, Dunlap NK. 2011. Sterol biosynthesis in the harmful marine dinoflagellate, Karenia brevis: Identification of biosynthetic intermediates produced during exposure to the fungicide fenpropidine. Phycological Research 59:54–63.

473 17. Leblond JD, Sabir K, Whittemore HL. 2023. Sterol Composition of the Peridinin-Containing
474 Dinoflagellate *Gertia stigmatica*, a Member of the Kareniaceae without a Canonical
475 Haptophyte-Derived Plastid. Protist 174:125939.

476 18. Mooney BD, Nichols PD, Salas MFD, Hallegraeff GM. 2007. LIPID, FATTY ACID, AND STEROL
477 COMPOSITION OF EIGHT SPECIES OF KARENIACEAE (DINOPHYTA): CHEMOTAXONOMY AND
478 PUTATIVE LIPID PHYCOTOXINS1. Journal of Phycology 43:101–111.

479 19. Place A, Harvey H, Bai X, Coats D. 2006. Sneaking under the toxin surveillance radar:
480 parasitism and sterol content. African Journal of Marine Science 28:347–351.

481 20. Santafé G, Paz V, Rodríguez J, Jiménez C. 2002. Novel Cytotoxic Oxygenated C29 Sterols from
482 the Colombian Marine Sponge *Polymastia t enax*. J Nat Prod 65:1161–1164.

483 21. Sun Y, Tian L, Huang J, Li W, Pei Y. 2006. Cytotoxic sterols from marine-derived fungus
484 Pennicillium sp. Natural Product Research 20:381–384.

485 22. Ishii T, Matsuura H, Zhaoqi Z, Vairappan CS. 2009. A New 4α-Methylated Sterol from a
486 Nephthea sp. (Nephtheidae) Bornean Soft Coral. 9. Molecules 14:3360–3366.

487 23. Akihisa T, Tanaka N, Yokota T, Tanno N, Tamura T. 1991. 5α-cholest-8(14)-en-3β-ol and three
488 24-alkyl-Δ8(14) -sterols from the bulbils of *Dioscorea batatas*. Phytochemistry 30:2369–2372.

489 24. Gawas D, Garcia R, Huch V, Müller R. 2011. A Highly Conjugated Dihydroxylated C28 Steroid
490 from a Myxobacterium. J Nat Prod 74:1281–1283.

491 25. Jackson CJ, Lamb DC, Marczylo TH, Warrilow AGS, Manning NJ, Lowe DJ, Kelly DE, Kelly SL.
492 2002. A Novel Sterol 14α-Demethylase/Ferredoxin Fusion Protein (MCCYP51FX) from

493 Methylococcus capsulatusRepresents a New Class of the Cytochrome P450 Superfamily *.
494 Journal of Biological Chemistry 277:46959–46965.

495 26. Li X, Roberti R, Blobel G. 2015. Structure of an integral membrane sterol reductase from
496 Methylomicrobium alcaliphilum. Nature 517:104–107.

497 27. Kim SW, Cha S-S, Cho H-S, Kim J-S, Ha N-C, Cho M-J, Joo S, Kim KK, Choi KY, Oh B-H. 1997.
498 High-Resolution Crystal Structures of Δ5-3-Ketosteroid Isomerase with and without a
499 Reaction Intermediate Analogue. Biochemistry 36:14030–14036.

500 28. Banta AB, Wei JH, Gill CCC, Giner J-L, Welander PV. 2017. Synthesis of arborane triterpenols
501 by a bacterial oxidosqualene cyclase. Proceedings of the National Academy of Sciences
502 114:245–250.

503 29. Bouvier P, Rohmer M, Benveniste P, Ourisson G. 1976. Delta8(14)-steroids in the bacterium
504 Methylococcus capsulatus. Biochem J 159:267–271.

505 30. Marijanovic Z, Laubner D, Möller G, Gege C, Husen B, Adamski J, Breitling R. 2003. Closing the
506 Gap: Identification of Human 3-Ketosteroid Reductase, the Last Unknown Enzyme of
507 Mammalian Cholesterol Biosynthesis. Mol Endocrinol 17:1715–1725.

508 31. Tsai P-L, Zhao C, Turner E, Schlieker C. The Lamin B receptor is essential for cholesterol
509 synthesis and perturbed by disease-causing mutations. eLife 5:e16011.

510 32. McCarty KD, Sullivan ME, Tateishi Y, Hargrove TY, Lepesheva GI, Guengerich FP. 2023.
511 Processive kinetics in the three-step lanosterol 14α-demethylation reaction catalyzed by
512 human cytochrome P450 51A1. Journal of Biological Chemistry 299.

513 33. Corey EJ, Virgil SC. 1991. An experimental demonstration of the stereochemistry of enzymic
514 cyclization of 2,3-oxidosqualene to the protosterol system, forerunner of lanosterol and
515 cholesterol. J Am Chem Soc 113:4025–4026.

516 34. Cho H-S, Choi G, Choi KY, Oh B-H. 1998. Crystal Structure and Enzyme Mechanism of Δ5-3-
517 Ketosteroid Isomerase from Pseudomonas testosteroni,. Biochemistry 37:8325–8330.

518 35. F Y, Jt B, Mm P, T D, As P, Js F, D H. 2020. Assessment of enzyme active site positioning and
519 tests of catalytic mechanisms through X-ray-derived conformational ensembles. Proceedings
520 of the National Academy of Sciences of the United States of America 117.

521 36. Akhtar M, Alexander K, Boar RB. 1978. Chemical and Enzymic Studies on the Characterization
522 of Intermediates during the Removal of the 14ac-Methyl-Group in Cholesterol Biosynthesis
523 169.

524 37. Chitwood DJ, Lusby WR, Lozano R, Thompson MJ, Svoboda JA. 1984. Sterol metabolism in the
525 nematodeCaenorhabditis elegans. Lipids 19:500–506.

526 38. Leblond JD, Chapman PJ. 2002. A Survey of the Sterol Composition of the Marine
527 Dinoflagellates Karenia Brevis, Karenia Mikimotoi, and Karlodinium Micrum: Distribution of
528 Sterols Within Other Members of the Class Dinophyceae1. Journal of Phycology 38:670–682.

529 39. García-Fernández E, Frank DJ, Galán B, Kells PM, Podust LM, García JL, Ortiz de Montellano
530 PR. 2013. A highly conserved mycobacterial cholesterol catabolic pathway. Environmental
531 Microbiology 15:2342–2359.

532 40. Holert J, Cardenas E, Bergstrand LH, Zaikova E, Hahn AS, Hallam SJ, Mohn WW. 2018.
533 Metagenomes Reveal Global Distribution of Bacterial Steroid Catabolism in Natural,
534 Engineered, and Host Environments. mBio https://doi.org/10.1128/mbio.02345-17.

535 41. Merino E, Barrientos A, Rodríguez J, Naharro G, Luengo JM, Olivera ER. 2013. Isolation of
536 cholesterol- and deoxycholate-degrading bacteria from soil samples: evidence of a common
537 pathway. Appl Microbiol Biotechnol 97:891–904.

538 42. Pandey AK, Sassetti CM. 2008. Mycobacterial persistence requires the utilization of host
539 cholesterol. Proceedings of the National Academy of Sciences 105:4376–4380.

540 43. Byskov AG, Andersen CY, Nordholm L, Thogersen H, Guoliang X, Wassmann O, Andersen JV,
541 Guddal E, Roed T. 1995. Chemical structure of sterols that activate oocyte meiosis. Nature
542 374:559–562.

543 44. Baltsen M, Byskov AG. 1999. Quantitation of meiosis activating sterols in human follicular
544 fluid using HPLC and photodiode array detection. Biomed Chromatogr 13:382–388.

545 45. Grøndahl C, Ottesen JL, Lessl M, Faarup P, Murray A, Grønvald FC, Hegele-Hartung C, Ahnfelt-
546 Rønne L. 1998. Meiosis-Activating Sterol Promotes Resumption of Meiosis in Mouse Oocytes
547 Cultured in Vitro in Contrast to Related Oxysterols. Biol Reprod 58:1297–1302.

548 46. Cavilla JL, Kennedy CR, Baltsen M, Klentzeris LD, Byskov AG, Hartshorne GM. 2001. The
549 effects of meiosis activating sterol on in-vitro maturation and fertilization of human oocytes
550 from stimulated and unstimulated ovaries*. Human Reproduction 16:547–555.

551 47. Guo R, Wang X, Li Q, Sun X, Zhang J, Hao R. 2020. Follicular fluid meiosis-activating sterol (FF-
552 MAS) promotes meiotic resumption via the MAPK pathway in porcine oocytes. Theriogenology
553 148:186–193.

554 48. Coticchio G, Rossi G, Borini A, Grøndahl C, Macchiarelli G, Flamigni C, Fleming S, Cecconi S.
555 2004. Mouse oocyte meiotic resumption and polar body extrusion in vitro are differentially
556 influenced by FSH, epidermal growth factor and meiosis-activating sterol. Hum Reprod
557 19:2913–2918.

558 49. Faerge I, Strejcek F, Laurincik J, Rath D, Niemann H, Schellander K, Rosenkranz C, Hyttel PM,
559 Grøndahl C. 2006. The effect of FF-MAS on porcine cumulus–oocyte complex maturation,
560 fertilization and pronucleus formation in vitro. Zygote 14:189–199.

561 50. Hegele-Hartung C, Kuhnke J, Lessl M, Grøndahl C, Ottesen J, Beier HM, Eisner S, Eichenlaub-
562 Ritter U. 1999. Nuclear and Cytoplasmic Maturation of Mouse Oocytes After Treatment with
563 Synthetic Meiosis-Activating Sterol In Vitro. Biol Reprod 61:1362–1372.

564 51. Bergh C, Group for the CIS, Loft A, Group for the CIS, Lundin K, Group for the CIS, Ziebe S,
565 Group for the CIS, Nilsson L, Group for the CIS, Wikland M, Group for the CIS, Gröndahl C,
566 Group for the CIS, Arce J-C, Group for the CIS. 2004. Chromosomal abnormality rate in
567 human pre-embryos derived from in vitro fertilization cycles cultured in the presence of
568 Follicular-Fluid Meiosis Activating Sterol (FF-MAS). Hum Reprod 19:2109–2117.

569 52. Hossein T, Shabani r, Artimani T, Pilehvari S, Moghimi M, Mehdizadeh M. 2024. Innovative
570 Approaches to Enhancing Oocyte Competence in patients with polycystic ovarian syndrome:
571 A Microfluidic Study with the Impact of FF-MAS on Rescued In Vitro Oocyte Maturation.
572 https://www.researchsquare.com. Retrieved 14 May 2024.

573 53. Blume T, Guttzeit M, Kuhnke J, Zorn L. 2003. Two syntheses of FF-MAS. Org Lett 5:1837–1839.

574 54. Zhou W, Fisher PM, Vanderloop BH, Shen Y, Shi H, Maldonado AJ, Leaver DJ, Nes WD. 2020. A
575 nematode sterol C4α-methyltransferase catalyzes a new methylation reaction responsible for
576 sterol diversity [S]. Journal of Lipid Research 61:192–204.

577 55. Giner J-L, Faraldos JA, Boyer GL. 2003. Novel sterols of the toxic dinoflagellate karenia brevis
578 (dinophyceae): a defensive function for unusual marine sterols?1. Journal of Phycology
579 39:315–319.

580 56. Van der Geize R, Yam K, Heuser T, Wilbrink MH, Hara H, Anderton MC, Sim E, Dijkhuizen L,
581 Davies JE, Mohn WW, Eltis LD. 2007. A gene cluster encoding cholesterol catabolism in a soil
582 actinomycete provides insight into Mycobacterium tuberculosis survival in macrophages.
583 Proceedings of the National Academy of Sciences 104:1947–1952.

584 57. Podust LM, Poulos TL, Waterman MR. 2001. Crystal structure of cytochrome P450 14α-sterol
585 demethylase (CYP51) from Mycobacterium tuberculosis in complex with azole inhibitors.
586 Proceedings of the National Academy of Sciences 98:3068–3073.

587 58. Jackson CJ, Lamb DC, Marczylo TH, Parker JE, Manning NL, Kelly DE, Kelly SL. 2003.
588 Conservation and cloning of CYP51: a sterol 14α-demethylase from *Mycobacterium*
589 *smegmatis*. Biochemical and Biophysical Research Communications 301:558–563.

590 59. Mohamed H, Child SA, Bruning JB, Bell SG. 2022. A comparison of the bacterial CYP51
591 cytochrome P450 enzymes from *Mycobacterium marinum* and *Mycobacterium tuberculosis*.
592 The Journal of Steroid Biochemistry and Molecular Biology 221:106097.

593 60. Eshraghisamani R, Mirto AJ, Wang J, Behr MA, Barkema HW, De Buck J. 2022. Identification of
594 essential genes in Mycobacterium avium subsp. paratuberculosis genome for persistence in
595 dairy calves. Front Microbiol 13.

596 61. Rifat D, Chen L, Kreiswirth BN, Nuermberger EL. 2021. Genome-Wide Essentiality Analysis of
597 Mycobacterium abscessus by Saturated Transposon Mutagenesis and Deep Sequencing.
598 mBio 12:10.1128/mbio.01049-21.

599 62. DeJesus MA, Gerrick ER, Xu W, Park SW, Long JE, Boutte CC, Rubin EJ, Schnappinger D, Ehrt S,
600 Fortune SM, Sassetti CM, Ioerger TR. 2017. Comprehensive Essentiality Analysis of the
601 Mycobacterium tuberculosis Genome via Saturating Transposon Mutagenesis. mBio
602 8:10.1128/mbio.02133-16.

603 63. Lee S-S, Young L-H, Wang KC. 1991. Microbial Transformation of Cycloartenol, 24-
604 Methylenecycloartanol, and Lanosterol, II. Isolation and Characterization of C_{19} Steroids. J Nat
605 Prod 54:178–183.

606 64. Wang KC, You B-J, Yan J-L, Lee S-S. 1995. Microbial Transformation of Lanosterol Derivatives
607 with Mycobacterium sp. (NRRL B-3805). J Nat Prod 58:1222–1227.

608 65. Araldi E, Fernández-Fuertes M, Canfrán-Duque A, Tang W, Cline GW, Madrigal-Matute J, Pober
609 JS, Lasunción MA, Wu D, Fernández-Hernando C, Suárez Y. 2017. Lanosterol Modulates TLR4-
610 Mediated Innate Immune Responses in Macrophages. Cell Reports 19:2743–2755.

611 66. Zimmermann M, Kogadeeva M, Gengenbacher M, McEwen G, Mollenkopf H-J, Zamboni N,
612 Kaufmann SHE, Sauer U. 2017. Integration of Metabolomics and Transcriptomics Reveals a

613 Complex Diet of Mycobacterium tuberculosis during Early Macrophage Infection. mSystems
614 2:10.1128/msystems.00057-17.

615 67. Cornejo-Granados F, López-Leal G, Mata-Espinosa DA, Barrios-Payán J, Marquina-Castillo B,
616 Equihua-Medina E, Zatarain-Barrón ZL, Molina-Romero C, Hernández-Pando R, Ochoa-Leyva
617 A. 2021. Targeted RNA-Seq Reveals the M. tuberculosis Transcriptome from an In Vivo
618 Infection Model. Biology 10:848.

619 68. Whittenbury R, Phillips KC, Wilkinson JF. 1970. Enrichment, Isolation and Some Properties of
620 Methane-utilizing Bacteria. Microbiology 61:205–218.

621 69. Li MZ, Elledge SJ. 2012. SLIC: A Method for Sequence- and Ligation-Independent Cloning, p.
622 51–59. *In* Gene Synthesis. Humana Press.

623 70. Bligh EG, Dyer WJ. 1959. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION.
624 Canadian Journal of Biochemistry and Physiology https://doi.org/10.1139/o59-099.

625 71. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped
626 BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids
627 Research 25:3389–3402.

628 72. Chen I-MA, Markowitz VM, Palaniappan K, Szeto E, Chu K, Huang J, Ratner A, Pillay M,
629 Hadjithomas M, Huntemann M, Mikhailova N, Ovchinnikova G, Ivanova NN, Kyrpides NC.
630 2016. Supporting community annotation and user collaboration in the integrated microbial
631 genomes (IMG) system. BMC Genomics 17:307.

632 73. Markowitz VM, Chen I-MA, Palaniappan K, Chu K, Szeto E, Pillay M, Ratner A, Huang J, Woyke
633 T, Huntemann M, Anderson I, Billis K, Varghese N, Mavromatis K, Pati A, Ivanova NN, Kyrpides

634 NC. 2014. IMG 4 version of the integrated microbial genomes comparative analysis system.
635 Nucleic Acids Research 42:D560–D567.

636 74. Katoh K, Standley DM. 2013. MAFFT Multiple Sequence Alignment Software Version 7:
637 Improvements in Performance and Usability. Mol Biol Evol 30:772–780.

638 75. Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple
639 sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066.

640 76. Minh BQ, Nguyen MAT, von Haeseler A. 2013. Ultrafast Approximation for Phylogenetic
641 Bootstrap. Molecular Biology and Evolution 30:1188–1195.

642 77. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R.
643 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the
644 Genomic Era. Molecular Biology and Evolution 37:1530–1534.

645 78. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. 2022. ColabFold:
646 making protein folding accessible to all. Nat Methods 19:679–682.

647 79. Guzenko D, Burley SK, Duarte JM. 2020. Real time structural search of the Protein Data Bank.
648 PLOS Computational Biology 16:e1007970.

649 80. Eisenberg D, Schwarz E, Komaromy M, Wall R. 1984. Analysis of membrane and surface
650 protein sequences with the hydrophobic moment plot. J Mol Biol 179:125–142.

651