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3 **Title**

4 A *cis*-prenyltransferase from *Methanosarcina acetivorans* catalyzes both head-to-tail

5 and non-head-to-tail prenyl condensation

6

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23 **Running title**

24 Irregular side reaction of a *cis*-prenyltransferase

Abbreviations

DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; HMBC, hetero-nuclear multiple-bond connectivity; HSQC, hetero-nuclear single quantum coherence; IPP, isopentenyl diphosphate; MOPS, 3-morpholinopropanesulfonic acid

Keywords

cis-prenyltransferase; archaea; glycosyl carrier lipid; isoprenoid; diterpenoid

Abstract

Cis-prenyltransferase usually consecutively catalyzes the head-to-tail condensation reactions of isopentenyl diphosphate to allylic prenyl diphosphate in the production of (*E,Z*-mixed) polyprenyl diphosphate, which is the precursor of glycosyl carrier lipids. Some recently discovered homologs of the enzyme, however, catalyze the non-head-to-tail condensation reactions between allylic prenyl diphosphates. In the present study, we characterize a *cis*-prenyltransferase homolog from a methanogenic archaeon, *Methanosarcina acetivorans*, to obtain information on the biosynthesis of the glycosyl carrier lipids within it. This enzyme catalyzes both head-to-tail and non-head-to-tail condensation reactions. The kinetic analysis shows that the main reaction of the enzyme is consecutive head-to-tail prenyl condensation reactions yielding polyprenyl diphosphates, while the chain lengths of the major products seem shorter than expected for the precursor of glycosyl carrier lipids. On the other hand, a subsidiary reaction of the enzyme, i.e., non-head-to-tail condensation between dimethylallyl diphosphate and farnesyl diphosphate, gives a novel diterpenoid compound, geranyllavandulyl diphosphate.

53 **Introduction**

54 *Cis*-prenyltransferases [1] catalyze consecutive head-to-tail condensation reactions of
55 isopentenyl diphosphate (IPP) to allylic prenyl diphosphate to produce the precursor of
56 glycosyl carrier lipids such as dolichol for eukaryotes and undecaprenol for bacteria,
57 which are required for N-linked protein glycosylation and peptide glycan biosynthesis,
58 respectively (Fig. 1). The intermediates and the final product of the reaction, i.e.,
59 (poly)prenyl diphosphates, are the oligomers/polymers of C_5 prenyl moieties, and the
60 prenyl moieties that are newly condensed by the action of the enzyme have a double
61 bond with *Z*-configuration. The primer substrate of the enzyme is usually (all-*E*) prenyl
62 diphosphate such as (*E,E*)-farnesyl diphosphate (FPP), in which case the intermediate of
63 the condensation reaction should be (*E,Z*-mixed) prenyl diphosphate. The intermediate
64 is used as an allylic substrate for the next condensation step with IPP to provide a longer
65 intermediate, and the condensation is repeated until the carbon chain of the product
66 finally reaches the required length, which can be anticipated from the chain lengths of
67 the glycosyl carrier lipids, e.g., C_{85-105} for human and C_{55} for general bacteria.

68 Recently, a few homologs of *cis*-prenyltransferase were shown to catalyze the
69 non-head-to-tail condensation between allylic prenyl diphosphates (Fig. 1). For example,
70 lavandulyl diphosphate synthase from lavender uses two dimethylallyl diphosphate
71 (DMAPP) molecules to yield a non-allylic product, lavandulyl diphosphate, which is a
72 precursor of a monoterpene alcohol, lavandulol [2]. Cyclolavandulyl diphosphate
73 synthase from *Streptomyces* sp. CL190 catalyzes both the same condensation between
74 two DMAPPs and an additional cyclization of the intermediate lavandulyl diphosphate
75 to give a non-standard allylic prenyl diphosphate, which is used for the modification of
76 a secondary metabolite to form lavanducyanin [3]. Another non-standard allylic prenyl

77 diphosphate, isosesquilavandulyl diphosphate, is synthesized from geranyl diphosphate
78 (GPP) and DMAPP by the action of a *cis*-prenyltransferase homolog from *Streptomyces*
79 sp. strain CNH-189 [4]. That compound is used for the biosynthesis of meroterpenoid
80 antibiotics, merochlorins. The substrate specificities of these enzymes are high: They do
81 not catalyze a standard head-to-tail condensation between allylic prenyl diphosphate and
82 IPP.
83 With the exception of a few previous characterization studies on the enzymes from
84 *Sulfolobus acidocaldarius* [5], *Aeropyrum pernix* [6], and *Thermococcus kodakarensis*
85 [7], which yield polyprenyl diphosphates with carbon chains up to C_{55-65}, archaeal
86 *cis*-prenyltransferases have not been sufficiently elucidated. The structures of glycosyl
87 carrier lipids also remain unclear in most archaeal species. The detailed structures have
88 been determined only for the lipids from *Halobacterium halobium* [8], *Haloferax*
89 *volcanii* [9, 10], *S. acidocaldarius* [11], and *Pyrococcus furiosus* [12] (Fig. 1). The chain
90 lengths of the lipids are C_{45-60}, which approximates their bacterial C_{55} counterpart. The
91 archaeal lipids, however, are classified as dolichols because their α-terminal prenyl
92 moiety is reduced. Interestingly, some of them also have reduced prenyl moieties at
93 their ω-termini [9-12]. On the other hand, older reports concerning glycosyl carrier
94 lipids from methanogenic archaea have suggested that both dolichol and polyprenol are
95 used in some species [13-15]. A methanogenic archaeon, *Methanosarcina acetivorans*,
96 possesses three *cis*-prenyltransferase homologs, which is exceptional. This situation
97 motivated us to characterize the homologs from *M. acetivorans* in the present study, and
98 in the process we encountered a *cis*-prenyltransferase that catalyzes both head-to-tail
99 and non-head-to-tail prenyl condensations.
100

101 **Results**

102 By searching databases such as KEGG (http://www.kegg.jp), we found three
103 *cis*-prenyltransferase homologs, MA1831, MA3723 and MA4402, encoded in the
104 genome sequence of *M. acetivorans*. Among them, MA3723 is the closest relative of the
105 previously characterized archaeal *cis*-prenyltransferases from *S. acidocaldarius* and *T.*
106 *kodakarensis*, while MA1831 is closest to the enzyme from *A. pernix*. A phylogenetic
107 analysis of *cis*-prenyltransferase homologs led to the construction of a phylogenetic tree
108 (Fig. 2) with three distinct (but not always monophyletic) clusters of the hypothetical
109 proteins from Euryarchaeota. A cluster colored in yellow, to which MA3723 belongs,
110 contains one homolog from each species of Euryarchaeota including *T. kodakarensis*,
111 suggesting that it is composed of the orthologs of the essential enzyme required for
112 glycosyl carrier lipid biosynthesis. Another cluster colored in light blue contains
113 MA4402 and its supposed orthologs found in the genomes of some limited
114 Euryarchaeota species. The third cluster forming an isolated, beige-colored branch is
115 composed of the orthologs of MA1831, which exist only in *Methanosarcina* spp. The
116 *cis*-prenyltransferases from *Saccharomyces cerevisiae*, *Escherichia coli*, and the two
117 species of Crenarchaeota, *S. acidocaldarius* and *A. pernix*, independently take deeply
118 branched positions.

119 We constructed the pET32a-based *E. coli* expression system of each homolog
120 from *M. acetivorans* to obtain recombinant proteins as the fusion with an N-terminal
121 Thioredoxin-His_6-S-tag. MA1831 and MA4402 were efficiently expressed in *E. coli*
122 and were purified using a polyhistidine tag, while the major portion of MA3723
123 seemingly formed an inclusion body even with the coexpression of chaperons, leaving
124 only a small portion in the purified fraction (Fig. 3A-C). In a prenyltransferase assay

125 using ^{14}C-labeled IPP and an allylic prenyl diphosphate [DMAPP, GPP, FPP or
126 geranylgeranyl diphosphate (GGPP)], only MA1831 catalyzed the condensation of IPP
127 with prenyl diphosphates longer than C_5. Although the purified MA1831 fraction
128 contained some protein contaminants, the affinity-purified fraction from the mock cells,
129 which contained similar contaminants and was used as the negative control, did not
130 show prenyltransferase activity. The enzyme did not accept DMAPP as an allylic
131 substrate, which resembles the substrate specificity of *cis*-prenyltransferases that are
132 responsible for glycosyl carrier lipid biosynthesis [1]. MA3723 and MA4402 were
133 completely inactive, even when they were mixed or coexpressed. We cannot exclude the
134 possibility that the addition of the long (>160 a.a.) N-terminal tag inactivates MA3723
135 and MA4402, while such N-terminal tagging was utilized in our previous study on
136 *cis*-prenyltransferase (and also for MA1831 in the present work) without causing
137 inactivation [6]. Given these conditions, we started the characterization of the
138 recombinant MA1831 in order to obtain information on the biosynthesis of the glycosyl
139 carrier lipid in *M. acetivorans*.
140 Reversed phase TLC analyses of the products from the prenyltransferase
141 reactions with MA1831 were performed after the acid phosphatase treatment of them. It
142 should be noted that Triton X-100 was at first added in these reactions because a
143 detergent is sometimes required as the activator of *cis*-prenyltransferases [16, 17]. The
144 major products from these standard prenyl condensation reactions using the allylic
145 substrate (GPP, FPP or GGPP) and IPP with the same concentration had a chain length
146 of C_{30} (Fig. 4A). The chain lengths of the products were, however, still shorter than
147 those of the glycosyl carrier lipids from *M. acetivorans*. As mentioned in our previous
148 paper [18], we detected undecaprenol and dihydroundecaprenol (though the position of

149 the double bond reduction was unclear) via LC-MS analysis of the lipid that was
150 extracted from *M. acetivorans* and then treated with phosphatase (Fig. 5). These C_{55}
151 polyprenols are considered to be the glycosyl carrier lipids of the archaeon because the
152 lipid from a relative species, *Methanosarcina barkeri*, also has been reported as
153 undecaprenol [15]. To establish the chain length of the final product of MA1831, we
154 raised the ratio of IPP against the allylic substrate to 10, and removed Triton X-100,
155 which generally activates *cis*-prenyltransferases by enhancing turnover, but at the same
156 time causes the production of shorter products [19, 20]. As a result, the chain lengths of
157 the major products increased to C_{35-45} (Fig. 4B), which was still shorter than C_{55}.
158 Moreover, we tried the removal of the N-terminal tag from the recombinant MA1831 to
159 investigate its effect on product chain length. Enterokinase treatment at 22°C of the
160 fusion enzyme, however, resulted in inactivation of the prenyltransferase. Therefore, the
161 gene of MA1831 was subcloned into a pET48b(+) vector because the fusion protein
162 obtained from the system can be cleaved by human rhinovirus 3C protease at 4°C. By
163 the treatment with the protease (Fig. 3D), an N-terminal Thioredoxin-His_6-tag was
164 removed without a significant loss of MA1831 activity. The tag-free MA1831 was
165 purified and used for prenyltransferase assay under the same conditions, i.e., 10 times
166 larger amount of IPP against that of an allylic substrate, without Triton X-100, and so on.
167 The removal of the tag slightly elongated the chain lengths of major products to C_{35-50}
168 (Fig. 4C). The formation of a C_{55} product, i.e., undecaprenyl diphosphate, was observed
169 only when GPP was used as the allylic substrate. The longest products, however, were
170 shorter than C_{55} when FPP and GGPP were the allylic substrate.
171 Next, as a trial, we examined the activity of MA1831 to catalyze non-standard
172 reactions that included a non-head-to-tail prenyl condensation between allylic prenyl

173 diphosphates. First, ^{14}C-labeled FPP was reacted with the same amount of an allylic
174 substrate using either DMAPP, GPP, FPP, or GGPP in the absence of Triton X-100.
175 Only the combination of FPP and DMAPP gave a new product with a chain length that
176 was estimated to be C_{20} because its R_f value approximated that of geranylgeraniol in the
177 reversed-phase TLC analysis (Fig. 6A). The chain length did not change when the ratio
178 of DMAPP against FPP was increased to 10 (Fig. 6B). The product from FPP and
179 DMAPP was also analyzed via normal-phase TLC. A new major product migrated
180 slightly farther than FPP, but was distinctly separated from prenyl alcohols and prenyl
181 monophosphates, suggesting that the product had a diphosphate group (Fig. 6C).
182 When ^{14}C-labeled GGPP was used, faint spots of new products were observed when
183 DMAPP or GPP was used as the counter substrate (Fig. 6D). These results demonstrated
184 that MA1831 accepts DMAPP as the prenyl acceptor substrate alternative to IPP, when
185 FPP is the prenyl donor substrate. It should be noted that the activity of non-head-to-tail
186 prenyl condensation between FPP and DMAPP was also observed with the tag-free
187 MA1831 derived from the pET48b-based system (data not shown), while all the data
188 shown above were obtained using the fusion enzyme from the pET32a-based system.
189 To establish the compatibility of the non-standard reaction with a standard
190 head-to-tail condensation, kinetic analyses of the N-terminal tagged MA1831 were
191 performed in the absence of Triton X-100. To assay the standard reaction, we quantified
192 the radioactivity incorporated from [^{14}C]IPP into the mixture of hydrophobic products.
193 The K_m for IPP was 7.21 μM when the concentration of FPP was maintained at 100 μM,
194 while the V_{max} was 4.71 nmol IPP·min^{-1}·mg protein^{-1}. We also observed the inhibitory
195 effect of DMAPP against the standard reaction to roughly estimate the affinity of the
196 enzyme to DMAPP, which acts as a mixed inhibitor, as shown in Fig. 7, not just as a

197 competitor against IPP. DMAPP itself is not a preferred prenyl donor substrate for
198 MA1831 and thus unlikely to compete with FPP, but it is conceivable that the product of
199 the non-head-to-tail condensation between FPP and DMAPP competes with FPP. In the
200 interest of simplification, however, the K_i of DMAPP was calculated using an equation
201 for competitive inhibition against IPP. In the presence of 150 μM DMAPP (and 100 μM
202 FPP), the K_m' for IPP was increased to 12.2 μM, while the V_{max}' was decreased to 2.92
203 nmol IPP·min^{-1}·mg protein^{-1}. The calculated K_i of DMAPP was 217 μM, which was
204 much larger than the K_m and K_m' for IPP. Moreover, the specific activity of MA1831
205 was measured using 5 μM ^{14}C-labeled FPP. When 100 μM IPP was used as the counter
206 substrate, the specific activity was 4.77 nmol IPP·min^{-1}·mg protein^{-1}, which almost
207 approximated the V_{max} of the standard reaction. This result suggested that the
208 concentration of FPP was sufficiently higher than its K_m, which seemed below 1 μM but
209 could not be determined accurately because the radioactivity of the products
210 incorporated from ^{14}C-labeled IPP was too low when FPP was added at lower
211 concentrations where a Michaels and Menten curve would be given. When the counter
212 substrate was changed to 400 μM DMAPP, the concentration exceeding its K_i, the
213 specific activity of the non-standard reaction was 0.477 nmol DMAPP·min^{-1}·mg
214 protein^{-1}, which was 10-fold smaller than the standard reaction. These data clearly
215 showed that the non-standard reaction between FPP and DMAPP was a side reaction,
216 and that the main reaction of MA1831 was the standard head-to-tail condensation
217 between the allylic substrates and IPP.

218 Such substrate specificity, however, has never been reported for usual
219 *cis*-prenyltransferases, nor for their homologs that catalyze only non-head-to-tail prenyl
220 condensations. Thus, the structure of the irregular product of MA1831 obtained from

221 the subsidiary non-standard reaction between FPP and DMAPP attracted our interest.
222 The LC-ESI-MS analysis of the product gave a negative ion with an *m/z* value that was
223 equal to that of GGPP, while the retention time of the product was slightly different
224 from that of GGPP (Fig. 8). This demonstrated that the product was an isomer of GGPP.
225 Then we hydrolyzed the product with acid phosphatase and purified the resultant
226 alcohol using HPLC. As outlined in Figs. 9-11 and Table 1, ^{1}H
227 and ^{13}C-NMR, ^{1}H-^{1}H-COSY, ^{1}H-^{13}C-edited-hetero-nuclear single quantum coherence
228 (HSQC), and ^{1}H-^{13}C-hetero-nuclear multiple-bond connectivity (HMBC) analyses gave
229 structural information that was sufficient to allow us to determine that the alcohol was
230 geranyllavandulol. Therefore, we concluded that the irregular product of MA1831 is
231 geranyllavandulyl diphosphate. The structure suggests that the reaction mechanism of
232 the non-head-to-tail condensation between FPP and DMAPP that is catalyzed by
233 MA1831 (Fig. 12) resembles that of lavandulyl diphosphate synthase.
234
235

236 **Discussion**

237 Among the three *cis*-prenyltransferase homologs from *M. acetivorans*, only
238 MA1831 showed enzymatic activity. This result suggests that MA1831 is responsible
239 for the biosynthesis of glycosyl carrier lipids in *M. acetivorans*. Nevertheless, the major
240 products from the standard head-to-tail prenyl condensation reactions catalyzed by
241 MA1831 were, under all reaction conditions we used for the *in vitro* assays, shorter than
242 undecaprenyl diphosphate, which is the supposed precursor of C_{55} glycosyl carrier
243 lipids in *M. acetivorans*. A slight amount of the C_{55} product was produced only when
244 GPP was used as the allylic substrate against a 10-fold larger amount of IPP, but was not
245 produced when the allylic substrate was either FPP or GGPP, which are general
246 precursors for glycosyl carrier lipids. Furthermore, the probable orthologs of MA1831
247 are possessed only by some species of genus *Methanosarcina* among the archaea, and
248 also by bacterial species of class Clostridia, which implies that the ancestral gene of the
249 archaeal MA1831 orthologs might have emerged by horizontal gene transfer. Under
250 these circumstances, we cannot exclude the possibility that one of the other
251 *cis*-prenyltransferase homologs, i.e., MA3723 or MA4402, is responsible for the
252 biosynthesis of glycosyl carrier lipids, whereas neither of them showed activity in the
253 present study for unknown reasons. Recently, the heteromer formation of
254 *cis*-prenyltransferase in animals and yeast was reported [21]. The heteromeric enzymes
255 are composed of a subunit similar to the usual homodimeric *cis*-prenyltransferases and
256 another subunit that is homologous to Nogo-B receptor, which is a transmembrane
257 protein containing a *cis*-prenyltransferase-like domain. Such a heteromeric enzyme
258 system also exists in some higher plants and is involved in dolichol and rubber
259 biosynthesis [22, 23]. Our phylogenetic analysis revealed that the organisms possessing

260 the probable ortholog of MA3723 always have a probable MA4402 ortholog and that
261 the cluster containing MA4402 is somewhat related to *S. cerevisiae* NUS1, the Nogo-B
262 receptor-like subunit of dehydrodolichyl diphosphate synthase. Based on the notion that
263 MA4402 and MA3723 might also form a heteromer to exert enzyme activity, we mixed
264 the separately purified proteins and also constructed their co-expression system, in
265 which each protein was expressed in *E. coli* as a fusion with the N-terminal tag. Both of
266 these trials, however, did not yield active *cis*-prenyltransferase (data not shown).

267 MA1831 also catalyzes the non-head-to-tail prenyl condensation between FPP
268 and DMAPP, probably as a side reaction. To our knowledge, the product
269 geranyllavandulyl diphosphate is a novel compound, and the geranyllavandulyl
270 structure has never been observed in natural compounds. It should be mentioned,
271 however, that geranyllavandulyltoluquinol has been reported as the hypothetical
272 precursor of irregular diterpenoid toluquinols, i.e., neobalearone and epineobalearone,
273 from the brown alga *Custoseira amentacea* var. *stricta* [24, 25]. Geranyllavandulyl
274 diphosphate is, however, unlikely to be the precursor of these natural compounds
275 because it does not possess the allylic diphosphate structure needed for prenyl donor
276 substrates. Instead, the precursor could be geranylisolavandulyl diphosphate if the
277 prenyltransfer reaction to toluquinol occurs singly, unlike the biosynthesis of
278 sophoraflavanone G that requires two dimethylallyltransferases to yield a lavandulyl
279 group [26]. The fact that geranyllavandulyl diphosphate cannot be a prenyl donor
280 suggests that the non-head-to-tail prenyl condensation catalyzed by MA1831 probably
281 has no physiological meaning. Such irregular prenyl chains, however, can extend the
282 structural variety of natural isoprenoids by substituting their regular prenyl groups, and
283 thus will be valuable for the synthesis of novel compounds. The characteristic amino

284 acid sequence of MA1831 that causes the low specificity toward prenyl acceptor
285 substrates, i.e., IPP for head-to-tail condensation and DMAPP for non-head-to-tail
286 condensation, remains unclear. In the future, however, mutagenic and structural studies
287 should uncover the specific traits of the *cis*-prenyltransferase homologs that catalyze
288 non-head-to-tail prenyl condensations such as lavandulyl diphosphate synthase,
289 cyclolavandulyl diphosphate synthase, isosesquilavandulyl diphosphate synthase, and
290 MA1831. Moreover, it seems important to determine whether the previously reported
291 *cis*-prenyltransferases also catalyze such non-standard reactions.
292

293 **Materials and Methods**

294 Materials

295 Precoated reversed-phase thin-layer chromatography (TLC) plates, RP18, and
296 normal-phase TLC plates, Silica gel 60, were purchased from Merck Millipore,
297 Germany. [1-^{14}C]IPP was purchased from GE Healthcare, UK. Non-labeled IPP,
298 DMAPP and FPP were donated by Dr. Chikara Ohto, Toyota Motor Co. GPP was
299 donated by Drs. Kyozo Ogura and Tanetoshi Koyama, Tohoku University. GGPP was
300 purchased from Larodan fine chemicals, Sweden.

301

302 Database search and phylogenic analysis

303 A database search for the homologs of known *cis*-prenyltransferases was performed
304 using a web service provided by KEGG. The multiple alignment of archaeal
305 *cis*-prenyltransferase homologs was performed using the Clustal Omega program [27]
306 provided at the EMBL-EBI website (http://www.ebi.ac.uk/). The phylogenetic tree was
307 constructed via the neighbor joining method using the CLC Sequence Viewer, ver. 7.5
308 (CLC bio, Denmark).

309

310 Expression of recombinant *cis*-prenyltransferase homologs from *M. acetivorans*

311 The genes of the *cis*-prenyltransferase homologs from *M. acetivorans*, *ma1831*,
312 *ma3723* and *ma4402*, were amplified from genomic DNA using KOD DNA polymerase
313 (TOYOBO, Japan), and the primer pairs are listed below (the recognition sequences of
314 the restriction enzymes are underlined): for *ma1831*,
315 5'-gt<u>ccatgg</u>acatggatacacctaaatttaaaagac-3' and 5'-atg<u>ctcgag</u>ttatccgcccaatgtaatatcttg-3';
316 for *ma3723*, 5'-at<u>ccatgg</u>acatggtaccgtggaataaaggagc-3' and

317 5’-gacctcgagagacttcaggccactccctg-3’; and, for *ma4402*,
318 5’-gtccatggacatggacctgctttcttttggtc-3’ and 5’-agtctcgagtcactttccgtaccttctctgc-3’. The
319 amplified DNA fragments were digested with *Nco*I and *Xho*I, and each of the digested
320 fragments was ligated with an *Nco*I/*Xho*I-digested pET32a(+) expression vector
321 (Novagen, USA) to construct pET32a-MA1831, pET32a-MA3723, and
322 pET32a-MA4402, respectively. For the subcloning of *ma1831* into pET48b(+)
323 (Novagen), the gene was amplified from the pET32a-MA1831 plasmid using the
324 primers 5’-ctctttcagggacccatggatacacctaaatttaaaagac-3’ and
325 5’-cggatcctggtacccttatccgcccaatgtaatat-3’. The amplified DNA fragment was ligated
326 with an *Sma*I-digested pET48b(+) vector to construct pET48b-MA1831 using an
327 In-Fusion advantage PCR cloning kit (TaKaRa, Japan) following the manufacturer’s
328 instructions. For the construction of the coexpression system of MA3723 and MA4402,
329 the *ma3723* gene, with the gene region encoding the N-terminal
330 Thioredoxin-His$_6$-S-fusion tag and containing the upstream ribosome-binding site, was
331 amplified from pET32a-MA3723 using the primer pair
332 5’-cggaaagtgactcgaaaggagatatacatatgagcg-3’ and
333 5’-ggtggtggtgctcgatcaggccactccctgcttttttaac-3’. The amplified DNA and *Xho*I-digested
334 pET32a-MA4402 were ligated to construct pET32a-MA4402/3723, in which *ma4402*
335 and *ma3723* compose an artificial operon, using an In-Fusion advantage PCR cloning
336 kit. After construction of the plasmids, the sequences of the inserted genes were verified
337 by DNA sequencing.
338 The plasmids constructed as described above were introduced into adequate *E. coli*
339 host strains such as BL21(DE3), BL21(DE3) transformed with a chaperon-expression
340 plasmid pG-KJE8 (Clontech, Japan), and KRX (Promega, USA). BL21(DE3) was

341 transformed with pET32a-MA1831 or pET48b-MA1831 and cultured in 300 mL LB
342 medium supplemented with 100 mg/L ampicillin at 37°C until formation of the log
343 phase, which was followed by overnight cultivation at 25°C after the addition of 0.2
344 mM IPTG. BL21(DE3)/pG-KJE8 was transformed with pET32a-MA3723 and cultured
345 in 300 mL LB medium containing 100 mg/L ampicillin, 20 mg/L chloramphenicol, 0.4
346 g/L L-arabinose, and 5 µg/L tetracycline. The culture was grown at 37°C until log phase,
347 and additional cultivation was performed at 22°C overnight after the addition of 0.1 mM
348 IPTG. KRX was transformed with pET32a-MA4402 and cultured in 300 mL LB
349 medium supplemented with 100 mg/L ampicillin at 37°C until log phase, and then at
350 22°C overnight after the addition of 0.1% L-rhamnose. The cultivation of KRX
351 transformed with pET32a-MA3723/4402 was performed under the same conditions
352 used for KRX/pET32a-MA4402.
353 The cells harvested from the culture were disrupted by sonication using a UP200S
354 ultrasonic homogenizer (Hielscher Ultrasonics, Germany) in a HisTrap binding buffer
355 (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH7.4). The homogenate
356 was centrifuged at 24,000 g for 30 min, and the supernatant was recovered as a crude
357 extract. The crude extract was loaded onto a 1 mL HisTrap crude FF column (GE
358 Healthcare). The column was washed with 10 mL of the HisTrap binding buffer
359 (imidazole concentration was raised to 60 mM as needed), and the recombinant proteins
360 were eluted from the column with 5 mL of a HisTrap elution buffer (20 mM sodium
361 phosphate, 0.5 M NaCl, 500 mM imidazole, pH7.4). For the cleavage of a
362 Thioredoxin-His_6-tag, which was fused at the N-terminal of MA1831 purified from
363 BL21(DE3)/pET48b-MA1831, 300 µg of the purified fusion enzyme was reacted with 5
364 units of polyhistidine-tagged human rhinovirus 3C protease (Funakoshi, Japan)

365 overnight at 4°C, after buffer exchange into 50 mM Tris-HCl, pH8.0, containing 150
366 mM NaCl. The solution of the protease reaction was loaded on a 1 mL HisTrap crude
367 FF column equilibrated with the HisTrap binding buffer, and the flow-through fraction
368 was used as the solution of purified tag-free MA1831. The level of purification was
369 confirmed via SDS-PAGE.
370
371 Prenyltransferase assay and radio-TLC analysis of the products
372 A reaction mixture typically contains, in a total volume of 200 μL, 0.5 or 5 nmol
373 [^{14}C]IPP (2.04 GBq/mmol or 81.4 MBq/mmol, respectively), 0.5 nmol allylic prenyl
374 diphosphate (DMAPP, GPP, FPP or GGPP), 20 μmol 3-morpholinopropanesulfonic
375 acid (MOPS)-NaOH, pH7.0, 1 μmol $MgCl_2$, 0 or 0.1% Triton X-100, and 30-1,000
376 pmol of the purified *cis*-prenyltransferase homolog. After incubation at 40°C for 1 hr,
377 the reaction was stopped by chilling. After the addition of 200 μL water saturated with
378 NaCl, the hydrophobic products were extracted from the mixture with 600 μL 1-butanol
379 equilibrated against saturated saline. Exclusively for the kinetic analyses, the butanol
380 layer was washed with the same volume of saturated saline equilibrated against
381 1-butanol. Radioactivity in an aliquot of the butanol layer was measured using an
382 LSC-5100 liquid scintillation counter (Aloka, Japan). An unusual unit, nmol
383 IPP·min^{-1}·mg protein^{-1}, was used to express the specific activity of *cis*-prenyltransferase
384 because this assay method gives the amount of IPP incorporated into the mixture of
385 hydrophobic products rather than the amount of the products. Kinetic parameters were
386 obtained via Lineweaver-Burk linear regression analysis. The remainder of the butanol
387 extract was provided for product analysis via the radio-TLC method described below.
388 The non-head-to-tail prenyl condensation catalyzed by MA1831 was assayed

389 using ^{14}C-labeled FPP and GGPP, which were synthesized using FPP synthase from
390 *Geobacillus stearothermophilus* and GGPP synthase from *S. acidocaldarius*,
391 respectively, as described elsewhere [18]. The assay mixture typically contained, in a
392 total volume of 200 μL, ~0.15 nmol of the ^{14}C-labeled allylic prenyl diphosphate (FPP
393 or GGPP), 0.15 nmol of non-labeled allylic prenyl diphosphate (DMAPP, GPP, FPP or
394 GGPP), 20 μmol MOPS-NaOH, pH7.0, 1 μmol $MgCl_2$, and 30-400 pmol of purified
395 MA1831 fused with the N-terminal tag. After 1 hr incubation at 40°C, the hydrophobic
396 products and substrates were extracted with 1-butanol as described above to be used in
397 radio-TLC analysis.
398 For reversed-phase TLC analysis, the butanol extract containing radiolabeled
399 products/substrates was treated overnight with 2 mg of potato acid phosphatase (Sigma
400 Aldrich, USA) in a 2:1 mixture of methanol and 1 M sodium acetate buffer, pH4.6,
401 following a method developed by Fujii et al. [28]. The resultant prenyl alcohols were
402 extracted with *n*-pentane and analyzed with a RP18 reversed-phase TLC plate
403 developed with acetone/H_2O (9:1). For normal-phase TLC analysis, the butanol extract
404 was evaporated without phosphatase treatment, and then the residue was dissolved to
405 methanol and analyzed with a Silica gel 60 normal-phase TLC plate developed with
406 methanol/chloroform/5 mM NH_4HCO_3 (4:6:0.9). The distribution of radioactivity on
407 the plate was visualized with a Typhoon FLA7000 multifunctional scanner (GE
408 Healthcare) and, if required, quantified using the attached software Image Quant TL to
409 calculate the specific activities based on the consumption of the prenyl-donor substrates,
410 either IPP or DMAPP.
411
412 LC-ESI-MS analysis of the product from a non-standard MA1831 reaction

413 The reaction mixture for the preparation of a non-head-to-tail condensation product
414 of MA1831 contained, in a final volume of 300 µL, 20 nmol DMAPP, 20 nmol FPP, 30
415 µmol MOPS-NaOH, pH7.0, 0.3 µmol $MgCl_2$, and 0.4 nmol purified MA1831 fused
416 with the N-terminal tag. After incubation for 1 hr at 40°C, the reaction was stopped by
417 chilling. After the addition of 300 µL water saturated with NH_4HCO_3, the hydrophobic
418 products/substrates were extracted with 600 µL 1-butanol equilibrated against water.
419 The butanol layer was evaporated under a N_2 stream, and the residue was dissolved
420 with methanol/acetonitrile (1:1) to be used in LC-ESI-MS analysis.
421 LC-ESI-MS analysis was performed with an Esquire 3000 ion trap system (Bruker
422 Daltonics, USA) connected to an Agilent 1100 Series HPLC (Agilent Technologies,
423 USA). The compounds eluted from a COSMOSIL packed column $5C_{18}$-AR-II (2.0ID ×
424 150 mm, Nacalai, Japan) were detected via UV absorption at 210 nm and ESI-MS in the
425 negative mode. The mobile phase used for the analysis was made up of a mixture of
426 solution A (25 mM NH_4HCO_3) and B (acetonitrile) at a flow rate of 0.2 $mL \cdot min^{-1}$.
427 Sample elution was performed with the program as follows: the ratio of B was 10% for
428 the initial 3 min; a linear gradient from 10 to 100% B for the next 15 min; and, 100% B
429 for an additional 10 min. After each analysis, the column was equilibrated with 10% B.
430 The MS parameters were the same as those used in our previous work [29]: sheath gas,
431 N_2 of 30 psi; dry gas, N_2 of 7.0 L·min-1, 320°C; scanning range, 50-1,000 *m/z*; scan
432 speed, 13,000 $m/z \cdot sec^{-1}$; ion charge control target, 20,000; maximum accumulation time,
433 100 ms; averages, 10; and, rolling averaging, 2.
434

435 NMR analysis of the MA1831 product

436 The reaction mixture contained, in a final volume of 20 mL, 8 µmol DMAPP, 2

437 μmol FPP, 1 mmol MOPS-NaOH, pH7.0, 20 μmol $MgCl_2$, and 42 nmol purified
438 MA1831 fused with the N-terminal tag. After 3 hr incubation at 40°C, 20 mL of 1 M
439 sodium acetate buffer, pH4.6, containing 40 mg potato acid phosphatase was added.
440 The same reaction was performed in a total of 6 tubes at 37°C overnight. The
441 hydrolyzed product was extracted from the mixture with the same volume, and then
442 again with a half volume of *n*-pentane. The pentane layer was merged, and evaporated
443 under a N_2 stream. The residue was dissolved with 2 mL methanol. Typically, 100 μL
444 of the methanol solution was provided for HPLC separation performed with a Shimadzu
445 HPLC system equipped with a COSMOSIL Packed Column 5C_{18}-AR-II (10ID × 150
446 mm, Nacalai, Japan). The mobile phase was methanol/H_2O (9:1), and the flow rate was
447 3 mL·min^{-1}. The column temperature was kept at 40°C. The UV absorption of
448 carbon-carbon double bonds was monitored at 210 nm, and a peak fraction was
449 recovered. This separation process was repeated ~20 times, and the recovered fractions
450 were merged. The fraction was evaporated, and the residue was dissolved with 500 μL
451 CD_3OD. This evaporation-dissolution process was repeated twice for complete
452 substitution of the solvent with CD_3OD. ^{1}H and ^{13}C NMR, ^{1}H-^{1}H-COSY,
453 ^{1}H-^{13}C-edited-HSQC, and ^{1}H-^{13}C-HMBC analyses of the sample were performed with
454 an AVANCE III HD 600 NMR spectrometer equipped with a cryoprobe (600 MHz,
455 Bruker).
456
457 Structural investigation of glycosyl carrier lipids from *M. acetivorans*
458 Total lipids were extracted via a method established by Bligh and Dyer [30]
459 from ~3 g of *M. acetivorans* cells cultivated as described elsewhere [18]. The cells were
460 suspended in 15 mL of water, and 70 mL of methanol and 37.5 mL of chloroform were

461 added. The mixture was stirred for 2 hr, and then 37.5 mL each of chloroform and water

462 were added. After being stirred briefly, the mixture was allowed to settle and separate

463 into two phases. The aqueous layer was extracted again with 10 mL each of chloroform

464 and water. To the combined organic layer, an equivalent volume of methanol and an

465 80% volume of water were added and stirred, and the mixture was separated into two

466 phases. After the organic layer was evaporated to dryness at 55 □C under a stream o

467 nitrogen gas, the residual was dissolved with 4 mL of methanol/chloroform (1:1) and

468 mixed with an excess volume of cold acetone to be placed in the dark at 4 □C for 3 hr

469 The insoluble matter collected by centrifugation and decantation was dissolved with 0.5

470 mL of 1-butanol and subjected to a phosphatase treatment as described above. The

471 hydrolysate was extracted with *n*-pentane, evaporated to dryness, and again subjected to

472 acetone precipitation. After centrifugation to remove insoluble matter, the supernatant

473 was evaporated and dissolved to methanol/2-propanol (1:1) for LC-ESI/MS analysis.

474 The analysis was performed under the same conditions described above, with the

475 exception of the mobile phase being replaced with methanol/2-propanol/100 mg·L^{-1}

476 sodium acetate (9:10:1).

477

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481

482 **Author contributions**

483 TO and HH planned the experiments; TO, KE and KK performed the experiments;

484 TO, KE, KK, TY and HH analyzed the data; and, HH wrote the paper.

485

486

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583

584 **Table 1. ^{13}C and ^{1}H NMR assignments for geranyllavandulol**

position	δ_c	δ_H (multiplicity, *J* in Hz)
1	63.7	3.52 (dd, 10.8, 18.5), 3.53 (dd, 10.8, 19.8)
2	49.9	2.25 (m)
3	27.9	2.04 (m), 2.21 (m)
4	122.4	~5.12 (m)
5	135.3	
6	39.4	~2.00 (m)
7	~26	~2.10 (m)
8	124.0	~5.12 (m)
9	134.4	
10	39.4	~2.00 (m)
11	~26	~2.10 (m)
12	124.0	~5.12 (m)
13	130.7	
5-methyl	14.8	1.63 (s)
9-methyl	14.7	1.61 (s)
ω-*cis*	16.3	1.62 (s)
ω-*trans*	24.5	1.69 (s)
1’	145.6	
2’	111.31	4.75 (m), 4.84 (m)
1’-methyl	18.65	1.70 (s)
OH		4.64 (broad)

585

586

587 **Figure legends**

588

589 **Figure 1. Reactions catalyzed by *cis*-prenyltransferase and its homologs**

590 Some natural compounds biosynthesized from the products of the enzymes are also

591 exemplified.

592

593 **Figure 2. Phylogenetic tree of *cis*-prenyltransferase homologs**

594 The enzymes reported to have activity as listed below are colored in red: ApeUPS,

595 *cis*-prenyltransferase from *A. pernix*; SacUPS, from *S. acidocaldarius*; TkoUPS, from *T.*

596 *kodakarensis*; EcoUPS, undecaprenyl diphosphate synthase from *E. coli*; SceRER2 and

597 SceSRT1, dehydrodolichyl diphosphate synthases from *Saccharomyces cerevisiae*; and,

598 SceNUS1, a heteromer subunit of *S. cerevisiae* dehydrodolichyl diphosphate synthase.

599 Only SceNUS1 did not show enzyme activity independently. All other proteins colored

600 in blue are *cis*-prenyltransferase homologs from Euryarchaeota: AF0707 and AF1219

601 (nowadays, AF_RS03590 and AF_RS06170, respectively), from *Archaeoglobus*

602 *fulgidus*; Hvo_2315 and Hvo_2318, from *Haloferax volcanii*; MM_0014, MM_0618

603 and MM_1083, from *Methanosarcina mazei*; MBUR_0041, from *Methanococcoides*

604 *burtonii*; MCP_1817 and MCP_2939, from *Methanocella paludicola*; MTHE_0589 and

605 MTHE_1499, from *Methanosaeta thermophila*; MJ_1372, from *Methanocaldococcus*

606 *jannaschii*; MTH232, from *Methanothermobacter thermautotrophicus*; and, PH1590,

607 from *Pyrococcus horikoshii*. The homologs from *M. acetivorans*, MA1831, MA3723

608 and MA4402, are colored in green. Bootstrap values over 70% were indicated at each

609 node.

610

611 **Figure 3. SDS-PAGE analysis of the purified recombinant proteins from *E. coli***

612 **cells**

613 (A) MA1831 expressed in BL21(DE3)/pET32a-MA1831. (B) MA3723 expressed in

614 BL21(DE3)/pG-KJE8/pET32a-MA3723. (C) MA4402 expressed in

615 KRX/pET32a-MA4402. (A-C) Lanes 1 and 2, pellet and soluble fractions from the

616 centrifugation of lysed *E. coli* cells, respectively; lanes 3-5, flow-through, wash and

617 elution fractions from a HisTrap affinity column, respectively. The expected band of the

618 recombinant protein in the elution fraction (and also that in the pellet fraction if

619 necessary) is indicated with an asterisk. (D) The cleavage of fusion MA1831. Lane 1,

620 MA1831 fused with the N-terminal Thioredoxin-His_6-tag, which was purified from in

621 BL21(DE3)/pET48b-MA1831; lane 2, MA1831 solution after reaction with human

622 rhinovirus 3C protease; lane 3, flow-through fraction from a HisTrap affinity column,

623 containing tag-free MA1831 indicated with an asterisk.

624

625 **Figure 4. Radio-TLC analysis of the products from standard reactions of MA1831**

626 Reversed-phase TLC analyses were performed for the hydrolyzed products from

627 head-to-tail prenyl condensation reactions. The enzymes used were: for (A and B),

628 MA1831 fused with an N-terminal Thioredoxin-His_6-S-tag, obtained from the

629 pET32a-based expression system; and for (C), MA1831 purified after the removal of an

630 N-terminal Thioredoxin-His_6-tag, obtained from the pET48b-based system. The allylic

631 substrates shown below the figures were reacted with the same amount (A) or with a

632 10-fold larger amount (B and C) of ^{14}C-labeled IPP, in the presence (A) and absence (B

633 and C) of 0.1% Triton X-100. s.f., solvent front; ori., origin

634

635 **Figure 5. LC-ESI-MS analysis of glycosyl carrier lipids from *M. acetivorans***

636 The UV chromatograms of (A) authentic polyprenols mainly containing undecaprenol

637 (C_{55}, open triangle) and dodecaprenol (C_{60}) and (B) the phosphatase-treated total lipid

638 extracted from *M. acetivorans*. The peak co-eluted with authentic undecaprenol and an

639 ion with *m/z* of 790.1 is indicated by an open triangle, and another peak co-eluted with

640 an ion with *m/z* of 792.1 is indicated by a closed triangle. (C and D) Extracted ion

641 chromatograms with indicated *m/z* ranges. The *m/z* values of 790.1 and 792.1

642 correspond to $[M+Na]^+$ of undecaprenol and dihydroundecaprenol, respectively.

643

644 **Figure 6. Radio-TLC analysis of the products from non-standard reactions of**

645 **MA1831**

646 (A) Reversed-phase TLC analysis for the hydrolyzed products from non-head-to-tail

647 prenyl condensation between ^{14}C-labeled FPP and the same amount of a non-labeled

648 allylic substrate shown in the figure. An arrowhead indicates the spot of a new product

649 from a non-head-to-tail condensation between FPP and DMAPP. (B) Reversed-phase

650 TLC analysis of the hydrolyzed products from the reaction between ^{14}C-labeled FPP

651 and DMAPP with various ratios shown in the figure. (C) Normal-phase TLC analysis of

652 the products from the reaction between ^{14}C-labeled FPP and DMAPP, without

653 phosphatase treatment. An arrowhead indicates the spot of the new product. (D)

654 Reversed-phase TLC analysis of the hydrolyzed products from the reaction

655 between ^{14}C-labeled GGPP and the same amount of a non-labeled allylic substrate

656 shown in the figure. Arrowheads emphasize the faint spots of probable reaction products.

657 s.f., solvent front; ori., origin

658

659 **Figure 7. Lineweaver-Burk plots for the determination of K_m for IPP and V_{max}**

660 Head-to-tail condensation reactions against 100 μM FPP were performed in the absence

661 and presence of 150 μM DMAPP.

662

663 **Figure 8. LC-ESI-MS analysis of the product from MA1831-catalyzed**

664 **non-head-to-tail prenyl condensation between FPP and DMAPP**

665 (A) UV chromatograms at 210 nm of the hydrophobic substrates/products extracted

666 with 1-butanol from the reaction mixture containing MA1831 (upper panel) or not

667 (middle panel), and that of authentic GGPP (lower panel). An arrowhead indicates the

668 peak of a new product. (B) Full scan mass spectrum of the new product. The analysis

669 was performed in the negative-ion mode.

670

671 **Figure 9. NMR analyses of the hydrolyzed product from MA1831-catalyzed**

672 **non-head-to-tail prenyl condensation between FPP and DMAPP**

673 (upper panel) ^{1}H-NMR spectrum. (lower panel) ^{13}C-NMR spectrum. The atom

674 numbering is shown in Figure 11.

675

676 **Figure 10. 2D-NMR analyses of the hydrolyzed product from MA1831-catalyzed**

677 **non-head-to-tail prenyl condensation between FPP and DMAPP**

678 (upper panel) ^{1}H-^{1}H-COSY spectrum. (lower panel) ^{1}H-^{13}C-edited-HSQC spectrum.

679 The atom numbering is shown in Figure 11.

680

681 **Figure 11. 2D-NMR analyses of the hydrolyzed product from MA1831-catalyzed**

682 **non-head-to-tail prenyl condensation between FPP and DMAPP (continued)**

683 (upper panel) ^{1}H-^{13}C-HMBC spectrum. (lower panel) Selected COSY (red) and HMBC
684 (blue) data of the hydrolyzed product geranyllavandulol, with the numbering of the
685 atoms.
686
687 **Figure 12. Hypothetical reaction mechanism of a non-head-to-tail condensation**
688 **between FPP and DMAPP catalyzed by MA1831**
689 The specificity of proton abstraction by a base from the *E*- or *Z*-methyl group of
690 DMAPP is unclear now.
691