

# Evidence for farnesol-mediated isoprenoid synthesis regulation in a halophilic archaeon, *Haloferax volcanii*

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**Abstract** Farnesol strongly inhibited growth of a halophilic archaeon, *Haloferax volcanii*, with an  $IC_{50}$  value of only 2  $\mu$ M (0.4  $\mu$ g/ml) in rich medium and 50 nM (0.01  $\mu$ g/ml) in minimal medium without lysis. Other isoprenoid alcohols such as isopentenol, dimethylallyl alcohol, geraniol, and geranylgeraniol at 500  $\mu$ M did not affect its growth. Mevalonate, which is the precursor of all isoprenoid membrane lipids in archaea, led to recovery of the growth inhibition of *H. volcanii*, but acetate had no such effect. Farnesol inhibited incorporation of acetate, but not mevalonate, into the lipid fraction. These results suggest that farnesol inhibited the biosynthetic pathway from acetate (acetyl-CoA) to mevalonate. Farnesol is known to be derived from the important intermediate of isoprenoids, farnesyl diphosphate (FPP), and found in neutral lipid fraction from this archaeon. Moreover, the cell-free extracts from *H. volcanii* could phosphorylate farnesol with ATP to generate farnesyl monophosphate and FPP. We conclude that farnesol-mediated isoprenoid synthesis regulation system by controlling farnesol concentration is present in *H. volcanii*.

**Key words:** Isoprenoid synthesis regulation; Farnesol; Mevalonate synthesis; Archaea; *Haloferax volcanii*

## 1. Introduction

Isoprenoid synthesis is known to be essential for diverse cellular functions. Some of the more important products of the pathway are sterols, ubiquinones, dolichols, carotenoids, prenylated proteins, and archaeal membrane lipids [1–4]. All membrane glycerolipids of archaea are isoprenoid ether lipids, in contrast to the fatty acid ester ones occurring in eubacteria and eukaryotes. Several isoprenoid biosynthetic enzymes of archaea have been studied: 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [5], geranylgeranyl diphosphate (GGPP) synthase [6–9], farnesylgeranyl diphosphate synthase [10], and geranylgeranylglyceryl phosphate synthase which synthesizes an ether linkage between glycerol and isoprenoid alcohol [4]. On isoprenoid regulation in archaea, Cabrera et al. [11] reported the regulation of HMG-CoA reductase activity in response to mevalonate availability using mevinolin, a potent inhibitor of HMG-CoA reductase, in a halophilic archaeon. To our knowledge, there has been no other report concerning the regulation of archaeal isoprenoid synthesis. We have demonstrated that GGPP synthase purified from the methanogenic archaeon, *Methanobacterium thermoformicum*, synthesizes

both farnesyl diphosphate (FPP) and GGPP in ratios dependent on certain conditions [6]. High GGPP concentration inhibited GGPP synthesis with GGPP synthase but not FPP synthesis. This led us to propose that GGPP synthase is one of the regulation points of isoprenoid synthesis in archaea [6,7]. Studies on the regulation system of archaea are needed not only for understanding archaeal lipid biosynthesis but also for consideration as a new model of the early stage of cholesterologenesis, as Yamagishi [12] suggested that an enzyme involved in cholesterologenesis of eukaryotes was derived from archaea.

In this study, we demonstrate the evidence that farnesol-mediated isoprenoid synthesis regulation system by controlling farnesol concentration is present in *H. volcanii*.

## 2. Materials and methods

### 2.1. Chemicals

Isopentenol, dimethylallyl alcohol, geraniol, farnesol, and geranylgeraniol were kind gifts from Kuraray. Squalene and DL-mevalonate were purchased from Sigma and Tokyo Kasei, respectively. [ $^{14}$ C]Acetate (2.09 MBq/mmol) and [ $^3$ H]farnesol (2.1 MBq/mmol) were products of American Radiolabeled Chemicals. DL-[ $^3$ H]mevalonate (1221 MBq/mmol) was a product of DuPont-NEN.

### 2.2. Growth of *H. volcanii*

The rich medium for *H. volcanii* IFO 14742 (=DSM 3757; strain DS-2) growth contained 5 g of yeast extracts, 4 g of KCl, 20 g of  $MgSO_4 \cdot 7H_2O$ , 13 g of  $MgCl_2 \cdot 6H_2O$ , 1 g of  $CaCl_2 \cdot 2H_2O$ , 0.2 g of  $NaHCO_3$ , 1 g of glucose, and 206 g of NaCl per liter, pH 7.0. The minimal medium, as described by Mevarech and Werczberger [13] with slight modifications, contained 206 g of NaCl, 36.9 g of  $MgSO_4 \cdot 7H_2O$ , 0.4 g of KCl, 0.3 g of  $NH_4Cl$ , 4.5 g of glycerol, 0.5 g of sodium succinate, 0.5 g of  $CaCl_2 \cdot 2H_2O$ , 0.2 g of  $K_2HPO_4$ , 1.6 mg of  $MnCl_2$ , 0.44 mg of  $ZnSO_4$ , 2.3 mg of  $FeSO_4$ , and 0.05 mg of  $CuSO_4$  per liter. The minimal medium was used only for determination of farnesol-induced growth inhibition, and the rich one for the other experiments. Isoprenoids and other compounds dissolved in ethanol were added to growth media at the same time of inoculation of *H. volcanii*. The final concentrations of ethanol were lower than 0.5%, which did not affect growth as determined by  $A_{660}$  measurement.

### 2.3. Incorporation of lipid precursors into lipids with farnesol treatment

At 2 h from addition of 0–20  $\mu$ M farnesol, the radioactive precursors of [ $^{14}$ C]acetate (185 kBq) and [ $^3$ H]mevalonate (185 kBq) were added to the *H. volcanii* cultures (10 ml). The cultures were incubated for 3 h at 30°C with shaking. The cells were harvested by centrifugation and washed with basal salt buffer containing 20% NaCl, 2%  $MgCl_2$ , 2% KCl, and 50 mM Tris (pH 7.0). Total lipids were extracted from the washed cells by the Brigh and Dyer method [14]. The total lipid fraction in chloroform solution was washed with 2  $\times$  2 volumes of methanol/water mixture (1:0.9), and then counted for radioactivity with a scintillation counter (Packard Tri-Carb 460).

### 2.4. Farnesol phosphorylation assay

Cell-free extracts of *H. volcanii* were prepared by sonication and centrifugation. Assay mixture contained 50 mM Tris-HCl buffer (pH 7.0), 6 mM  $MgCl_2$ , 3.5 M KCl, 16 mM CHAPS, 10 mM ATP, 1  $\mu$ M [ $^3$ H]farnesol (37 kBq), and a suitable amount of the cell-free extracts in a final volume of 100  $\mu$ l. After incubation at 37°C for 1 h, the sample

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**Abbreviations:** HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate.

was extracted with 1-butanol. The sample was separated by TLC (Merck, TLC aluminium sheet) with a solvent system of 1-propanol/ ammonia water/water (6:3:1). The position of authentic standards, farnesyl monophosphate and FPP, were visualized with iodine vapor. Iodine-positive spots on the TLC sheet were cut down and counted for radioactivity with a liquid scintillation counter (Packard Tri-Carb 460).

### 3. Results and discussion

#### 3.1. Growth inhibition of *H. volcanii* by farnesol and other isoprenoid alcohols

Farnesol strongly inhibited the growth of the halophilic archaeon, *H. volcanii*, without lysis. The  $IC_{50}$  value of farnesol was only 2  $\mu$ M (0.4  $\mu$ g/ml) in rich medium and 50 nM (0.01  $\mu$ g/ml) in minimal medium (Fig. 1). Related isoprenoid alcohols, geranylgeraniol ( $C_{20}$ ), geraniol ( $C_{10}$ ), dimethylallyl alcohol ( $C_5$ ), and isopentenol ( $C_5$ ), did not inhibit growth at the concentration of 500  $\mu$ M (Table 1). Bradfute and Simoni [15] reported that the farnesol derivative farnesyl acetate has the farnesol-like activity of accelerating HMG-CoA reductase degradation. Farnesyl acetate was also tested against *H. volcanii*, but did not affect the growth at 100  $\mu$ M (Table 1). This organism probably has little activity of hydrolyzing the ester linkage of farnesyl acetate to produce farnesol, and thus farnesyl acetate has no farnesol-like activity against this archaeon.

#### 3.2. Prevention of farnesol-induced growth inhibition by mevalonate

Farnesol has been reported to accelerate HMG-CoA reductase degradation in animal cells [16]. Early studies using radiolabeled and  $^{13}C$ -labeled precursors showed that isoprenoids, polar lipids and squalene, in archaea [17–19] were synthesized from acetate and mevalonate as well as cholesterol in eukaryotes. Inhibition of HMG-CoA reductase reduces cholesterol synthesis in animal cells. Thus, in this archaeon, the isoprenoid precursors, mevalonate and acetate, were also expected to express the recovery activity. Mevalonate at 5 mM partially pre-

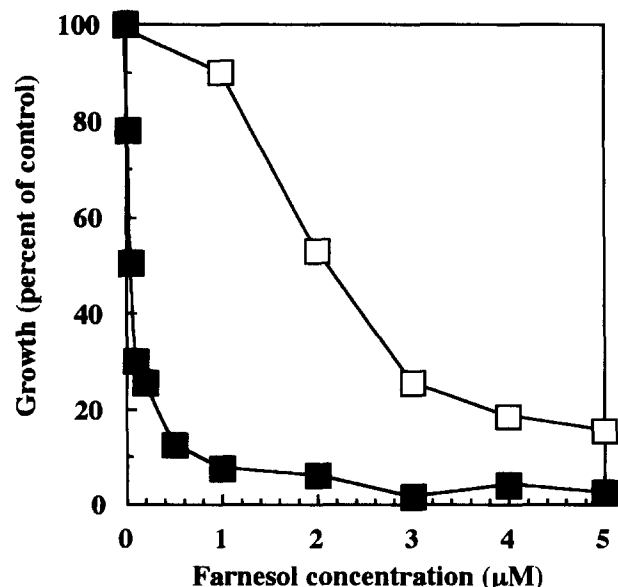


Fig. 1. Farnesol-induced growth inhibition of *H. volcanii*. *H. volcanii* was grown in rich medium (□) and in minimal medium (■) with farnesol at the indicated concentrations. Growth was measured by  $A_{660}$  at 2 days. Control culture had  $A_{660}$  of 0.8.

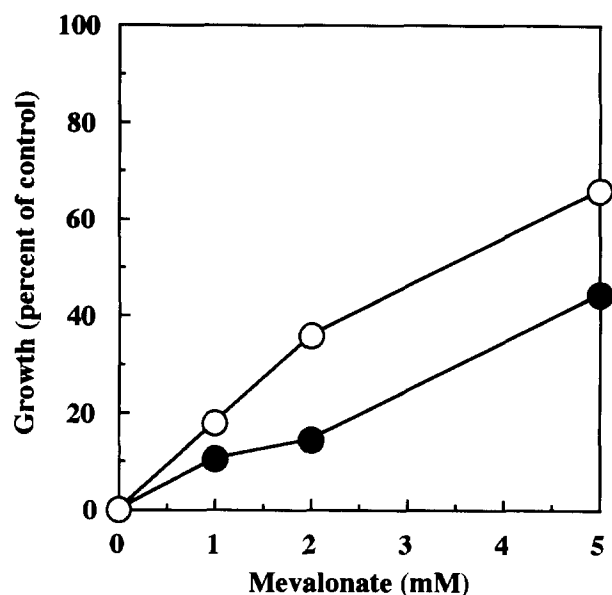


Fig. 2. Prevention of farnesol-induced growth inhibition of *H. volcanii* by mevalonate. *H. volcanii* was grown in rich medium containing farnesol at 10  $\mu$ M (○) and 20  $\mu$ M (●) with the indicated concentrations of mevalonate.

vented farnesol-induced growth inhibition (Fig. 2), while, acetate at 10 mM was not effective.

#### 3.3. Inhibition of incorporation of [ $^{14}C$ ]acetate into lipids by farnesol

Farnesol effect on incorporation of isoprenoid precursors, [ $^{14}C$ ]acetate and [ $^3H$ ]mevalonate, was investigated. Untreated cells (control) synthesized lipids using [ $^{14}C$ ]acetate and [ $^3H$ ]mevalonate (Fig. 3). Farnesol strongly inhibited incorporation of [ $^{14}C$ ]acetate, but not [ $^3H$ ]mevalonate, into the lipid fraction (Fig. 3). Farnesol did not inhibit acetate incorporation into trichloroacetic acid insoluble materials such as protein (data not shown). These results suggest that the farnesol-inhibition point occurs early in the isoprenoid synthesis pathway prior to mevalonate synthesis from acetyl-CoA. Moreover, farnesol inhibition of [ $^{14}C$ ]acetate incorporation was more stronger than

Table 1  
Effects of farnesol and related isoprenoid derivatives on growth of *H. volcanii*

Isoprenoid	Concentration ( $\mu$ M)	Growth (percent of control)
None	–	100
Isopentenol	100	101
	500	99
Dimethylallyl alcohol	100	103
	500	98
Geraniol	100	98
	500	97
Farnesol	1	90
	5	15
	10	0
Geranylgeraniol	100	108
	500	87
Farnesyl acetate	50	102
	100	86

*H. volcanii* was grown in rich medium for 2 days.

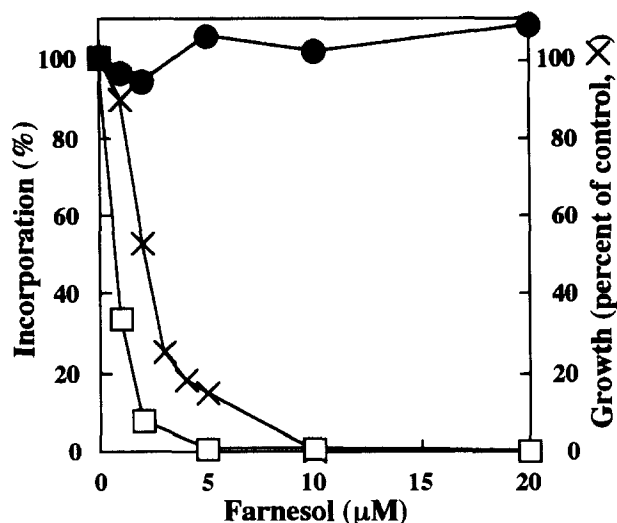


Fig. 3. Farnesol effect on incorporation of isoprenoid precursors. At 2 h from farnesol addition at the indicated concentrations, the radioactive precursors [<sup>3</sup>H]mevalonate and [<sup>14</sup>C]acetate were added to *H. volcanii* cultures in rich medium. The cultures were incubated for 3 h, and total lipids were extracted and counted for their radioactivities. The sample not treated with farnesol had 2,630 dpm for <sup>3</sup>H and 15,400 dpm for <sup>14</sup>C. Symbols indicate the following: (□), incorporation of [<sup>14</sup>C]acetate; (●), incorporation of [<sup>3</sup>H]mevalonate; (×), dose-response curve for farnesol-induced growth inhibition obtained from Fig. 1.

farnesol-induced growth inhibition, suggesting this is the primary action point of farnesol.

Edwards et al. [16,20] suggested that animal HMG-CoA reductase degradation occurs in the endoplasmic reticulum. The soluble HMG-CoA reductase of *H. volcanii* has significant homology with the C-terminal half including active domain of HMG-CoA reductases of animals, but not with transmembrane domains at the N-terminal [5]. At least, farnesol did not inhibit HMG-CoA reductase activity of *H. volcanii* (data not shown). Halobacteria have been reported to synthesize HMG-CoA through unusual pathway using acetyl-CoA and lysine [18], though the pathway is not characterized. In *H. volcanii*, farnesol might inhibit the pathway. Detailed farnesol inhibition mechanisms are now under investigation.

### 3.4. Farnesol phosphorylation by cell-free extracts

Evidence for farnesol phosphorylation has been obtained with algae [21]. Recently, in mammalian cells [22] and rat [23], farnesol has been reported to be utilized for cholesterol synthesis and protein isoprenylation, maybe after phosphorylation. Thermophilic archaeon, *Sulfolobus acidocaldarius*, possesses geranylgeraniol and farnesol phosphorylation enzymes (S. Ohnuma, personal communication). Cell-free extracts from

*H. volcanii* could also phosphorylate farnesol with ATP to produce farnesyl monophosphate and FPP as shown in Table 2.

In summary, in *H. volcanii*, farnesol inhibited early isoprenoid synthesis at a relatively low concentration. We and other investigators reported that archaea do not possess FPP synthase, and archaeal GGPP synthases synthesize both FPP and GGPP [6–9]. We demonstrated that using methanogen GGPP synthase, high concentration of GGPP inhibited GGPP synthesis, but not FPP synthesis [6]. If GGPP, a direct precursor of polar lipids, accumulates, it may cause FPP accumulation. Farnesol derived from FPP must finally accumulate in the cells and/or the culture. It was reported that squalene synthase-deficient mutant [24] and squalene synthase inhibitor [25] caused farnesol accumulation in cells and/or culture fluid. Farnesol is found in various archaea including *H. volcanii*. Moreover, farnesol itself was phosphorylated to generate FPP. Therefore, farnesol concentration can be controlled by phosphorylation and dephosphorylation by phosphatase. We propose that archaeal isoprenoid biosynthesis regulation by farnesol has following scenario: GGPP accumulation → FPP accumulation → farnesol production → reduction of mevalonate synthesis from acetyl-CoA (or → farnesol phosphorylation).

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Table 2  
Farnesol phosphorylation with cell-free extracts from *H. volcanii*

Substrate(s)*	Product (dpm)	
	Farnesyl monophosphate	FPP
[ <sup>3</sup> H]Farnesol + ATP	2,340	1,530
[ <sup>3</sup> H]Farnesol	0	0

\*Concentrations were described in section 2.

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