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Production of C_{35} isoprenoids depends on H_2 availability during cultivation of the hyperthermophile *Methanococcus jannaschii*

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Abstract A series of five progressively saturated C_{35} isoprenoids has been identified in cell-free extracts of the deep-sea methanogen *Methanococcus jannaschii*. Production and relative abundance of the isoprenoids were dependent on culture conditions; significant production occurred in a 16-l fermentor (12-l working volume) and a 2.5-l fermentor (2-l working volume) but could not be duplicated in serum bottles. Several factors were investigated and shown not to account for the different production levels, including medium composition, pH, and temperature. However, the interphase mass transfer rate was shown to significantly affect the production of C_{35} isoprenoids in a fermentor. The structures of the novel isoprenoids were confirmed by hydrogenation reactions and mass spectra of the isoprenoids. Indirect evidence based on genomics and mass spectrometry data implicates head-to-head condensation of farnesyl pyrophosphate (C_{15}) with geranylgeranyl pyrophosphate (C_{20}) as the mechanism for C_{35} synthesis.

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Introduction

Methanococcus jannaschii is a hyperthermophilic methanogen isolated from a hydrothermal vent at a depth of 2,610 m on the East Pacific Rise (Jones et al. 1983). *M. jannaschii* grows optimally near 85°C and displays a strong barophilic growth response when cultured in a stainless steel 316 high-temperature-pressure bioreactor at 86°C and 90°C (Kanseshiro and Clark 1995). It is a strict anaerobe, using H_2 and CO_2 as its sole substrates.

Membrane composition is one of the defining characteristics of the archaeal genus (van de Vossenberg et al. 1998). The two main components of archaeal membranes (Langworthy 1985) are ether-linked lipids and hydrophobic aliphatic isoprenoid chains. The isoprenoid chains are extremely stable over time, are found in a large variety of sediments, and have been used successfully as biomarkers to show archaeal activity during various geological periods (e.g., Wang 1998). Apart from *Methanosarcina barkeri* (Tornabene et al. 1979), which produces mainly C_{25} isoprenoids, the major hydrophobic components of methanogen membranes are C_{30} squalene and hydrosqualene derivatives (Tornabene et al. 1979). This is also true of *M. jannaschii*, where C_{30} isoprenoids have been reported to comprise 85% of the total isoprenoids (Comita et al. 1984).

In the present paper we describe the discovery of a previously unreported series of C_{35} isoprenoids produced by *M. jannaschii*. While some evidence has been reported for the existence of similar molecules in another hydrothermal vent methanogen (Holzer et al. 1988), we present here solid evidence for the structure of these novel molecules. Furthermore, production of the C_{35}s in *M. jannaschii* was dependent on the culture conditions; hydrogen availability in particular was shown to affect isoprenoid production.

Materials and methods

Microorganism and culture conditions

Methanococcus jannaschii JAL-1, DSM 2661, was obtained from David R. Boone (Oregon Collection of Methanogens, Portland, Ore.) and grown in serum bottles (165 ml) by strict anaerobic techniques, as described by Balch and Wolfe (1976). The defined medium contained the following components (g/l) (Tsao et al. 1994): $MgCl_2 \cdot 6H_2O$, 4.18; $MgSO_4 \cdot 7H_2O$, 3.4; KCl, 0.33; NH_4Cl, 0.25; K_2HPO_4, 0.14; $CaCl_2 \cdot 2H_2O$, 0.14; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.01; $NiCl_2 \cdot 6H_2O$, 0.0005; $Na_2SeO_3 \cdot 5H_2O$, 0.0003; resazurin, 0.001; NaCl, 30; $NaHCO_3$, 1.0 (or MES, 1.83, see Results and Discussion section for details); and $FeSO_4 \cdot 7H_2O$, 0.001. In addition, the medium contained 10 ml trace mineral solution (Zeikus 1977) and 10 ml vitamin solution. The vitamin solution consisted of the following (mg/l): biotin, 2.0; folic acid, 2.0; pyridoxine hydrochloride, 10.0; thiamine hydrochloride, 5.0; riboflavin, 5.0; nicotinic acid, 5.0; DL-calcium pantothenate, 5.0; vitamin B12, 0.1; *p*-aminobenzoic acid, 5.0; and lipoic acid, 5.0. The pH was adjusted to either 6.8 or 6.0 (see Results and Discussion section for details) with NaOH. Thirty milliliters of media was distributed into each serum bottle. The bottles were capped with crimped-in-place rubber stoppers and autoclaved. The gas atmosphere was flushed out with a gas mixture of H_2/CO_2 (4:1) before being reduced by addition of 2% (v/v) of $Na_2S \cdot 9H_2O$ and cysteine·HCl solutions (25 mg/ml). The bottles were inoculated with 3 ml (10%) of old cultures, and the pressure was then adjusted to 30 psi with the H_2/CO_2 gas mixture. Bottle cultivations were carried out at 78°C in either a shaking incubator (Model C24, New Brunswick Scientific, Edison, N.J.) at 200 rpm for variable amounts of time ranging from 6 to 12 h, or a reciprocal shaking water bath (Model 25, Precision Scientific, Chicago, Ill.) for 6 h at 200 rpm. Growth was monitored by removing 1 ml of medium at set intervals and measuring the optical density (OD) at 660 nm in a UV/Vis spectrophotometer.

The biomass from the controlled 12-l fermentation in complex medium was purchased from Eric Johnson (University of Illinois at Urbana-Champaign, Department of Microbiology, Urbana, Ill.). The detailed protocol for the fermentation can be found in Mukhopadhyay et al. (1999). Apart from scale, major differences with the serum bottle protocol described above are the addition of 2 g/l yeast extract and tryptone, growth temperature of 85°C, pressure of 15 psi, and continuous bubbling of H_2/CO_2 gas through the medium. Moreover, medium reduction was achieved by continuous addition of H_2S gas instead of a one-time addition of Na_2S.

Our fermentations were performed in a 2.5-l Bioflo III Batch/Continuous Fermentor (New Brunswick Scientific, Edison, N.J.), which was made airtight and fitted with an RL3 Externally Adjustable Relief Valve (Nupro, Willoughby, Ohio) to allow regulation of the pressure. The working volume was 2 l. Agitation was by means of two Rushton-type impellers (diameter 5.4 cm, distance between impellers 7.0 cm). The medium composition was the same as for the serum bottles, using 20 mM MES as buffer with the addition of 2 g/l of tryptone and yeast extract. Inoculum was grown in bottles as described. The fermentor and medium were autoclaved together and allowed to cool to 78°C before reduction and inoculation (10% v/v). The fermentor was then left overnight with gassing and agitation rates of 0.5 standard liters per minute (slpm) and 250 rpm, respectively. At these rates, the cells grew slowly and were poised for rapid growth when the higher rates of gassing (1 slpm) and agitation (400 rpm) were imposed in the morning. The pH was controlled through manual addition of NaOH.

Extraction of isoprenoids

M. jannaschii was harvested from cultures by centrifugation for 10 min at 8,000 *g* (4°C) and dried by lyophilization. The dried biomass was ground with a spatula, and 10 ml hexane was added per 0.1 g of dry cell weight. The hexane mixture was sonicated for 2 h and then left for 24 h at 40°C with shaking. The mixture was then filtered through a sintered glass filter. The hexane extract was dried with a gentle flow of nitrogen until the hexane completely evaporated. For the fermentor-grown biomass, 1.0 g of dried cells obtained from the University of Illinois was extracted with 200 ml of hexane.

Hydrogenation

Either 10 mg of pure squalene (Sigma S-3626) or 10 mg of the isoprenoid extract of *M. jannaschii* was dissolved in 1 ml ethyl acetate containing a few drops of acetic acid. PtO_2 (10 mole% with respect to substrate) was added to the reaction mixture as a catalyst, and a gas mixture of H_2/CO_2 (4:1) was supplied with a syringe until the pressure reached 30 psi. The reaction vial was sealed with a rubber stopper. The reactions were carried out for 6 h at room temperature using a magnetic stirrer.

Analysis

GC-MS was performed with a Hewlett Packard 6890 series gas chromatograph coupled to a Hewlett Packard Mass Selective Detector (5973 series) fitted with a 30 m (0.25 mm i.d., 0.25 μm film thickness) fused silica capillary column (DB-XLB, Agilent (J&W), Palo Alto, Calif.). The GC oven temperature was raised from 50°C to 250°C at 20°C/min after a 1-min hold at 50°C. The oven temperature was then maintained at 250°C for 10 min before being raised to the final temperature of 300°C at 20°C/min, where it was held for 20 min. Helium was the carrier gas with a linear velocity of 40 cm/s in the splitless mode. The mass spectrometer was operated in the electron impact ionization mode at an electron energy of 70 eV and an ion source temperature of 230°C, with scanning from 50 to 600 atomic mass units. Spectra were collected using HP Chemstation software. Before GC-MS analysis, the extracts were redissolved in 200 μl hexane per gram dry cell weight.

Pressure measurements in serum bottles

Pressure in serum bottles was measured by connecting a standard Ashcroft regulator manometer (0–100 psi) to a 1-cc syringe with flexible tubing. A needle was then attached to the syringe, enabling the measurement of pressure in the serum bottles while maintaining anaerobic conditions. The total volume of the syringe, tubing, and manometer system was approximately 1 ml; therefore, perturbation of the bottle pressure by measurement was very small. Testing with bottles of known pressures revealed that the precision of the pressure measurements was within 0.5 psi.

Calculation of the mass transfer rate k_La in serum bottles

The flow of hydrogen into the medium (per unit liquid volume), N_Ha, is given by (Blanch and Clark 1997):

$$N_H a = \frac{k_L}{H} a \cdot (p - H \cdot C) = -\frac{1}{V_L} \cdot \frac{dn}{dt} \quad (1)$$

where k_L is the liquid-phase mass transfer coefficient, a is the volumetric interfacial area, H is the Henry's constant, p is the partial pressure of H_2 in the headspace, C is the concentration of dissolved hydrogen, V_L is the liquid volume, t is time, and n is the number of moles of H_2 in the headspace. If the system is heavily mass transfer limited, then C can be neglected.

$$\frac{k_L}{H} a \cdot p = -\frac{1}{V_L} \cdot \frac{dn}{dt} \quad (2)$$

Considering H_2 to be an ideal gas and rearranging Eq. 2, one obtains

$$\frac{dp}{p} = -k_L a \cdot \frac{V_L}{V_G} \cdot \frac{R \cdot T}{H} \cdot dt \quad (3)$$

where R is the ideal gas constant (8.314 J/mol K), T is the temperature, and V_G is the headspace volume. Integration yields

$$\ln\left(\frac{p}{p_0}\right) = -k_L a \cdot \frac{V_L}{V_G} \cdot \frac{R \cdot T}{H} \cdot t \quad (4)$$

Henry's constant for hydrogen at 85°C is $H = 1.672 \times 105$ mol/m^3 Pa (Sander 1999). Thus,

$$\ln\left(\frac{p}{p_0}\right) = -3.880 \cdot 10^{-3} \cdot k_L a \cdot t \quad (5)$$

Results and discussion

Evidence of C_{35} isoprenoids

Figure 1A shows the gas chromatogram of a shaking-bottle culture extract. The five numbered peaks indicate the C_{30} isoprenoids. The decrease in molecular weight from one peak to the next is 2, corresponding to the dehydrogenation of a double bond; thus, the peaks are in order of increasing desaturation (the most saturated C_{30} isoprenoid is produced in minute amounts and is not indicated on this chromatogram). Fig. 1B corresponds to the fermentor-culture extract. The same series of C_{30} isoprenoids is apparent, albeit at much higher concentrations. Also present is another series of peaks. The difference in molecular weight between each of these new

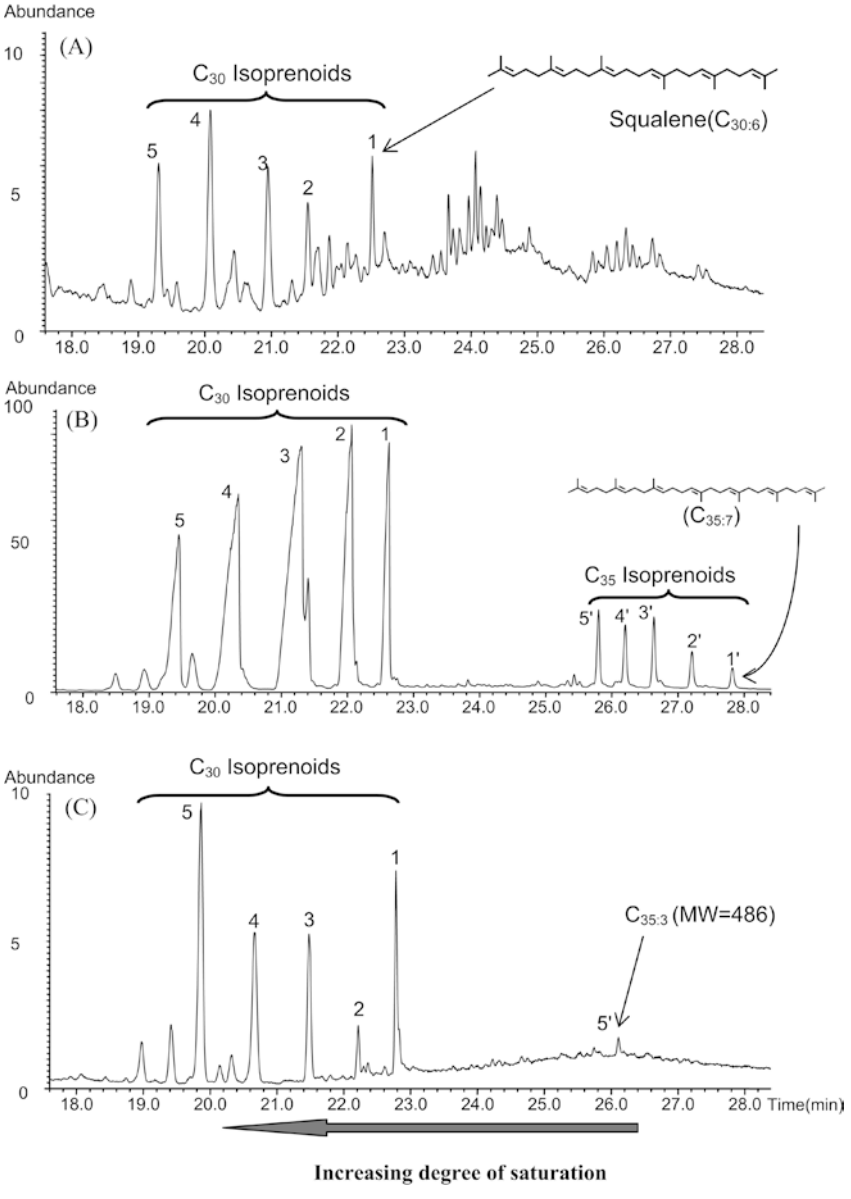

Fig. 1A–C Comparison of isoprenoid production under different growth conditions. **A** Non-repressurized serum bottles grown in orbital shaker. **B** 12-l fermentor culture. **C** Repressurized serum bottles grown in orbital shaker with MES buffer. 1 = $C_{30:6}$, 2 = $C_{30:5}$, 3 = $C_{30:4}$, etc., 1′ = $C_{35:7}$, 2′ = $C_{35:6}$, etc.

peaks and the corresponding C_{30} peaks is 68, equivalent to one IPP unit, indicating that these peaks are C_{35} analogues of the C_{30} series. Again, the two most saturated C_{35}s are produced in negligible quantities and are not indicated on the chromatogram.

Structures of the isoprenoids

The molecular weight, retention time, and mass spectrum of the C_{30} isoprenoid labeled 1 all indicate that it is squalene ($C_{30:6}$), produced by a head-to-head condensation of two farnesyl pyrophosphate (C_{15}) units. The C_{30} series therefore seems to be a progressively saturated group of squalenoids. This conclusion was confirmed via hydrogenation. As expected, the C_{30} series reduced to one compound, with a molecular weight, retention time, and mass spectrum matching that of squalane ($C_{30:0}$).

Similarly, the C_{35}s reduced to one compound upon hydrogenation, confirming that they also correspond to one series of progressively saturated isoprenoids. There are thus two options for the structure of the C_{35}s. Either they could be produced by a head-to-head condensation of one unit of FPP with one unit of geranylgeranyl pyrophosphate (GGPP, C_{20}), yielding a product analogous to squalene, or they could result from a head-to-tail condensation of seven IPP units, creating C_{35} equivalents of the regular C_{30} isoprenoids found in *Sulfolobus acidocaldarius* (Holzer et al. 1979). Two factors strongly support the first of these two hypotheses.

First, BLAST searches of the fully sequenced *Methanococcus jannaschii* genome revealed only one prenyltransferase-encoding gene. This has previously been annotated by The Institute for Genomic Research (Bult et al. 1996) as a bifunctional short-chain isoprenyl diphosphate synthase, closely resembling a prenyltransferase found in *Methanobacterium thermoautotrophicum* (Chen and Poulter 1993), which produces FPP and GGPP. No prenyltransferase capable of synthesizing longer isoprenyl chains was found.

Second, Holzer et al. (1979) have shown that mass spectral data can be used to distinguish between the two aforementioned cases. Cleavage occurs mainly next to tertiary carbons. Thus, for a fully saturated all-E (i.e., head-to-tail) C_{35} isoprenoid, one would expect major fragments at *m*/*e* 43, 57, 113, 127, 183, 197, 253, 267, 323, 337, 393, 407, 463, and 477, the MW of the unfragmented chain being 492. These ratios are organized in "doublets" (45 and 57, 113 and 127, and so on). Furthermore, the probability of formation of either fragment in a doublet is roughly the same, and the corresponding peaks should therefore have similar intensities. In contrast, a similarly saturated head-to-head structure is expected to yield fragments at *m*/*e* 43, 113, 183, 253, 267, 337, 407, 477, and the unfragmented 492. In this case, there is only one doublet (253 and 267), making the mass spectra easily differentiable. The mass spectrum of the *M. jannaschii* C_{35} clearly falls into this latter category, and the corresponding structures expected for the fully desaturated C_{35} isoprenoids are shown in Fig. 1.

Effect of culture conditions on *M. jannaschii* isoprenoid production

As can be readily seen from Fig. 1, isoprenoid production depends strongly on the culture conditions; they affect not only C_{35} isoprenoid production but also the relative distribution of the squalenoid series. In particular, tetrahydrosqualene ($C_{30:4}$, peak 3) has been reported as the main component in the membrane of *M. jannaschii* (Comita et al. 1984; Langworthy 1985); however, this is not the case in the serum-bottle extract presented in Fig. 1A, where hexahydrosqualene ($C_{30:3}$, peak 4) is the main product.

Extensive variations of the culture conditions in serum bottles, including media composition, pH, temperature, and agitation, yielded different main products, although in most cases octahydrosqualene ($C_{30:2}$, peak 5) was predominant. Small amounts of certain C_{35} isoprenoids (Table 1), primarily $C_{35:3}$ (peak 5′), were also produced (Fig. 1C). However, it was never possible

Table 1 Growth and C_{35} production of *Methanococcus jannaschii* in bottle culture relative to standard conditions (see Materials and Methods for further details)

Modification to culture conditions	Effect on growth	Effect on C_{35} production
Added yeast extract and tryptone (2 g/l)	Negligible	None[a]
Repressurized bottles every 3 h	Large increase in growth rate and final cell density	None
Lowered pH of unpressurized, room-temperature medium from 6.8 to 6.0	Negligible	Trace of $C_{35:3}$
Increased buffering (switched from $NaHCO_3$ to 10 mM MES)	Slight decrease in final cell density	$C_{35:3}$peak much clearer
Raised temperature from 78°C to 85°C (in MES buffer)	Shorter lag phase	No change
Switched salt solution to match Illinois' (see Materials and Methods section)	None	None
Combination of buffering, temperature and salt solution (as above)	Negligible	Observed trace amounts of $C_{35:3}$ and $C_{35:4}$
Changed from incubator shaker to reciprocal shaking water bath	Substantial increase in growth rate and final cell density	Observed trace amounts of $C_{35:3}$ and $C_{35:4}$

[a] "None" indicates no effect on C_{35} production and that no C_{35} isoprenoids were evident

to reproduce from bottle cultures the full spectrum of C_{35} isoprenoids, or even a squalenoid distribution resembling that obtained from the fermentor-culture extract.

Mass transfer limitation of hydrogen is a common problem in methanogen cultures, especially at high temperatures (Jud et al. 1997). This is also the case for *M. jannaschii*. Hydrogen availability has been shown to influence certain non-methanogenesis related functions in *M. jannaschii*, such as flagella production (Mukhopadhyay et al. 2000). A typical growth curve for incubator bottle cultures is shown in Fig. 2. Although growth is at first exponential, it quickly becomes linear, indicating mass transfer limited growth. The calculated mass transfer coefficients are consistent with the apparent mass transfer limitations of the incubator serum-bottle cultures: k_La in the Illinois fermentor was calculated to be roughly 1,400 h^{-1} (see Appendix), whereas k_La for the incubator-grown bottles was calculated to be 77.3 h^{-1} by measurement of the pressure drop.

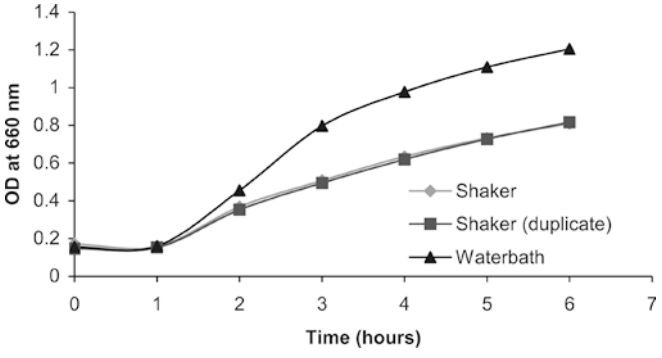

Fig. 2 Typical growth curves for orbital shaker and water bath (reciprocal shaking) cultures. Notice the linear aspect of the shaker cultures. Such growth curves are typical of mass transfer-limited growth

In an effort to overcome the mass transfer limitations evident in the incubator bottle cultures, cultures were also grown in a reciprocal shaking water bath. Stirring in the water bath proceeded via a more chaotic agitation of the culture than in the incubator, in which a more regular swirling motion of the culture fluid was observed. As evidenced by the comparison of growth curves in Fig. 2, growth was much faster in the water bath and followed a more exponential-like profile in the early stages of growth. Despite the apparent reduction of mass transfer limitations, no significant change in the production of C_{35} isoprenoids was observed (data not shown).

However, we were able to show conclusively in a 2.5-l fermentor that hydrogen availability significantly influences the production of C_{35} isoprenoids. Figure 3 shows the extracts from cultures grown in the fermentor with and without baffles. Addition of the baffles improves the mixing considerably, leading to an increase in k_La of 3- to 4-fold (see Appendix) without having a major effect on any other parameters. It can clearly be seen that the

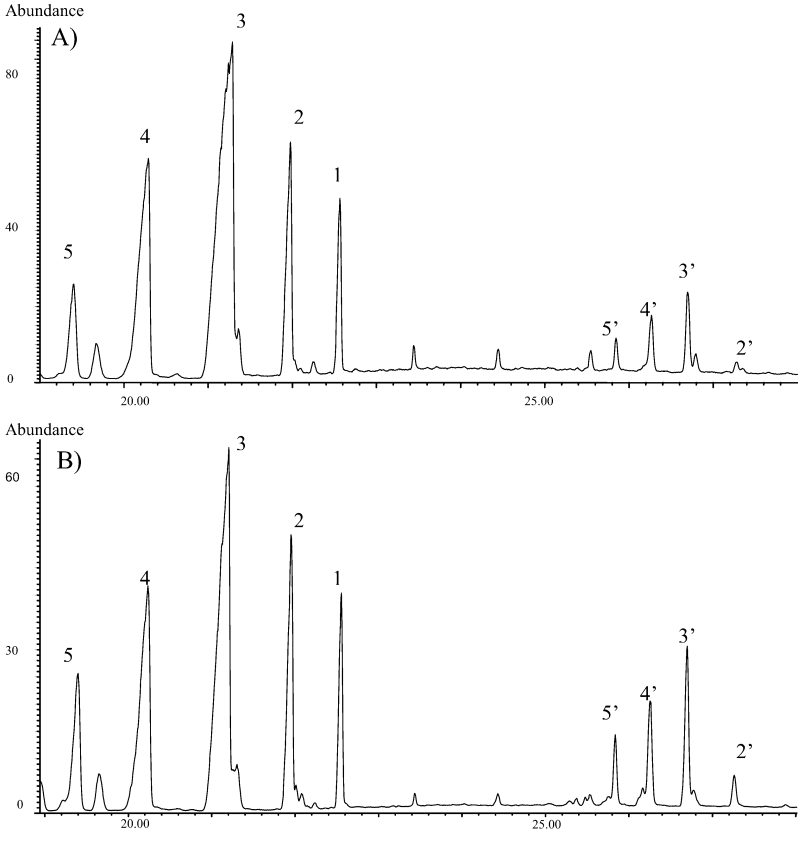

Fig. 3A, B Comparison of isoprenoid production in the small fermentor. **A** Unbaffled tank; **B** Tank baffled with four vertical baffles. The width of the baffles was 1.7 cm (1/8 of the tank diameter)

addition of baffles significantly increases the ratio of C_{35} to C_{30} isoprenoids.

It is interesting to note that, in our hands, there was always a clear correspondence between the distributions of C_{30} and C_{35} isoprenoids. In serum bottles the tetra-saturated species (peaks 5 and 5′) were predominant, whereas the fermentor cultures yielded a majority of the disaturated species (peaks 3 and 3′). Although this observation did not hold true for the samples from Illinois, it does suggest that the same mechanism is responsible for the hydrogenation of both systems. It is still not clear what causes the shift in distribution. Variations in the mass transfer rate in the fermentor did not affect the relative quantities of C_{30} isoprenoids produced.

One of the major differences between the bottles and fermentor, beyond the mass transfer rate, is the existence of a mechanical shear field in the fermentor due to the impeller. The occurrence of shear suggested that the shift to less-saturated isoprenoids might be due to a need to strengthen the cell membrane. However, no differences in the relative isoprenoid levels (beyond what could be attributed to the change in mixing) were observed when agitation rates were varied from 250 to 600 rpm (data not shown). Despite extensive variations of gassing and agitation in the 2.5-l fermentor, we could not obtain a C_{30} distribution similar to that in serum bottles.

Possible isoprenoid functions

Different functions have been suggested for the isoprenoids in archaeal membranes. Tornabene et al. (1978) proposed that they might function as reversible hydrogen sinks, thereby regulating the internal reduction potential of the cells. If this is the case, then H_2 mass transfer effects might be expected to play an important role in triggering the production of the C_{35} isoprenoids. The hydrogen concentrations in *M. jannaschii*'s natural habitat are highly variable (Jannasch and Mottl 1985), and it might be hypothesized that the cells use the isoprenoids to stock up on hydrogen when it is readily available. However, one would then expect also to see a shift towards more saturated species of both C_{30} and C_{35} isoprenoids. It seems likely that the influence of hydrogen availability is less direct.

Another possibility for the varying C_{35} production is that the cell changes the relative concentrations of the isoprenoids to regulate its membrane fluidity, in a process similar to homeoviscous adaptation in eubacteria (Sinensky 1974). The ether-linked lipid composition of *M. jannaschii* has previously been shown to vary in response to temperature (Sprott et al. 1991) and pressure (Kanseshiro and Clark 1995). The nonpolar components of the membrane lipids could be regulated similarly, and it would be interesting to compare production of polar and nonpolar lipids under a range of conditions.

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Appendix

Calculation of the mass transfer rate k_La in the 16-l fermentor

The following data apply to the 16-l baffled-tank fermentor used at the University of Illinois by E. Johnson (Mukhopadhyay et al. 1999):

Total volume (V_T): 16 l = $16{\cdot}10^{-3}$ m^3
Working volume (V_L): 12 l = $12{\cdot}10^{-3}$ m^3
Impeller speed (N) 600 rpm = 10 s^{-1}
Impeller diameter (D_I): 7.5 cm = 0.075 m (Rushton-type impeller)
Number of impellers (n): 3
Distance between impellers (ΔC): 10.2 cm = 0.102 m
Total gas flow (Q): 24 l/min = $4{\cdot}10^{-4}$m^3/s
The medium properties were taken to be the same as liquid water.
Density (ρ) 1,000 g/l = 1,000 kg/m^3 (at 20°C, little variation with temperature)
Viscosity (μ): 0.3396 g/m·s = $0.3396{\cdot}10^{-3}$ kg/m·s (at 85°C , CRC Handbook of Chemistry and Physics, online edition, http://www.hbcpnetbase.com)

The two main factors that affect k_La are the mixing and the gassing, which are measured in terms of the power input per unit liquid volume, W/V_L, and the superficial gas flow velocity, v_S. All equations are given in terms of SI units.

We start by determining the flow conditions in the fermentor. The Reynolds number, Re, in this case is defined as

$$\mathrm{Re} = \frac{D_I^2 \cdot N \cdot \rho}{\mu} \approx 1.66 \cdot 10^5 \tag{6}$$

A Reynolds number of 10^4 or more indicates a turbulent regime (Rushton et al. 1950). We can now determine the power input to the liquid, W. For a Rushton-type impeller under turbulent conditions, the dimensionless Power number, W^*, is constant and approximately equal to 6 in a baffled tank (1 for an unbaffled tank) (Dickey et al. 1976):

$$W^* = \frac{W}{\rho \cdot N^3 \cdot D_I^5} = 6 \tag{7}$$

However, this value holds only for a single impeller in a non-aerated vessel and must therefore be adjusted. Let us first consider the effect of multiple impellers. The power exerted by multiple impellers is closely linked to the distance that separates them. For spacing-to-diameter ratios $\Delta C/D_I$ larger or equal to 2–3 it can usually be assumed that the impellers act independently (Hudcova et al. 1989). Below this range, significant interactions begin to occur,

mostly resulting in solid-body rotations of the liquid located between the impellers. In this particular case,

$$\frac{\Delta C}{D_I} = 1.36 \tag{8}$$

Hudcova et al. (1989) have made extensive studies of the relationship between impeller spacing and power number for 2-impeller systems. Interpolation of their values yields $W^* \approx 10$ for dual impellers with a spacing-to-diameter ratio of 1.36. Assuming that the third impeller adds as much power as the second (a more conservative estimate than supposing each of the three impellers contributes the same power), we obtain a value for the power number $W^* \approx 14$.

The second factor we must take into consideration is the "aeration" (i.e., gassing) of the fermentor. The gas flowing through the fermentor will reduce the apparent density of the broth and thereby decrease the power input at a given agitation speed. This decrease is a function of gas flow rates, spacing-to-diameter ratios, and impeller geometry. First, we must verify that neither of the impellers is "flooded", i.e., that the gas is being effectively dispersed by the impellers and not simply flowing past it. This is especially critical for the lowest of the impellers. The transition between the two states is known as the flooding-to-loading transition (FLT) and can usually be determined by simple visual observation of the flow patterns. Flooding is not usually a problem in well-baffled small-scale systems as long as the agitation rate is kept above 200 rpm (for example, see data in Hudcova et al. 1989).

Because the gas is usually better dispersed in the upper levels of the fermentor, the decrease in the power supplied by the upper impellers is usually smaller than that for the lowest impeller. (In other words, the more impellers, the smaller the *average* power loss per impeller.) Once again, if the spacing-to-diameter ratio is large enough, the impellers can be treated independently. In this case, the lowest impeller can be treated as a single aerated impeller, while the others function as non-aerated impellers in a fluid with a density equal to the mean density of the gas-liquid mixture. For the lowest impeller, Hughmark (1980) gives

$$\left(\frac{W_{\text{gassed}}}{W_{\text{ungassed}}}\right)_{\text{low}} = 0.10 \cdot \left(\frac{Q}{N \cdot V_L}\right)^{-\frac{1}{4}} \left(\frac{N^2 \cdot D_I^4}{g \cdot b \cdot V_L^{\frac{2}{3}}}\right)^{-\frac{1}{5}} \tag{9}$$

where b is the width of the impeller blade.

The power input for an entire system of n impellers is given by:

$$\left(\frac{W_{\text{gassed}}}{W_{\text{ungassed}}}\right)_n = \frac{1}{n} \cdot \left\{ \left(\frac{W_{\text{gassed}}}{W_{\text{ungassed}}}\right)_{\text{low}} + (n-1)(1-\varepsilon_h) \right\} \tag{10}$$

where ϵ_h is the gas holdup, defined as the ratio of gas volume to liquid volume in the mixture. This is a generalization of the result for two impellers given by Nienow and Lilly (1979). Typically $\epsilon_h \approx 0.05$–0.15 and can be neglected as a first approximation.

In our case the impellers cannot be considered to be independent, and we must rely on empirical data. Hudcova et al. (1989) provided the following ratio for the 2-impeller system:

$$\left(\frac{W_{\text{gassed}}}{W_{\text{ungassed}}}\right)_n \approx 0.5 \tag{11}$$

Assuming this value holds for the 3-impeller system (as explained above, the actual value is probably higher), we obtain:

$$W = W^* \cdot \left(\frac{W_{\text{gassed}}}{W_{\text{ungassed}}}\right) \cdot \rho \cdot N^3 \cdot D_I^5 = 16.6 \text{ Watt} \tag{12}$$

The power input per liquid volume is thus:

$$\frac{W}{V_L} \approx 1.4 \text{kWatt}/\text{m}^3 \tag{13}$$

To calculate the superficial velocity of the gas, we need to know the surface area of the fermentor, A. Because the only information available is the total volume, an assumption must be made about the height-to-diameter ratio of the vessel, H/D. A standard value for this ratio is 2:1. Thus,

$$V_T = H \cdot A = 2D \cdot \pi \left(\frac{D}{2}\right)^2 = \frac{\pi}{2} \cdot D^3 = 16 \cdot 10^{-3} \text{m}^3 \tag{14}$$

and

$$D = 0.217 \text{ m} \tag{15}$$

This yields

$$A = \pi \left(\frac{D}{2}\right)^2 = \frac{\pi}{4} \left(\sqrt[3]{\frac{2}{\pi} V_T}\right)^2 = 3.69 \cdot 10^{-2} \text{m}^2 \tag{16}$$

The total gas flow, neglecting the H_2S, is $Q = 24$ l/min $= 4 \cdot 10^{-4} \text{m}^3/\text{s}$.

The superficial velocity is thus

$$v_S = \frac{Q}{A} = 1.08 \cdot 10^{-2} \text{m}/\text{s} \tag{17}$$

There are several correlations from which to calculate $k_L a$ for oxygen. One of the most commonly used for inviscid, non-coalescing systems (such as fermentation broths containing electrolytes) is given by van't Riet (1979), who reviewed a large number of correlations:

$$k_L a(O_2) = 2.0 \cdot 10^{-3} \cdot \left(\frac{W}{V_L}\right)^{0.7} \cdot v_S^{0.2} (\text{SI units}) \tag{18}$$

This relationship is valid (within 20–40%) for vessels between 2 and 4,400 l and for W/V_L values between 500 and 10,000 W/m^3.

The interfacial area per unit volume a can be assumed to be independent of the gas species. The value of $k_L a$ for hydrogen can thus be calculated by adopting a standard penetration model (Bird et al. 1960), which states that k_L is proportional to the square root of the diffusivity. Therefore,

$$k_L a(H_2) = 2.0 \cdot 10^{-3} \cdot \left(\frac{W}{V_L}\right)^{0.7} \cdot v_S^{0.2} \cdot \sqrt{\frac{D_{H_2}}{D_{O_2}}} \tag{19}$$

where $D_x$is the diffusivity of species x.

Diffusivities of gases are typically difficult to measure, particularly at high temperatures (CRC Handbook of Chemistry and Physics, online edition, http://www.hbcpnetbase.com). The approximate temperature dependence of the diffusivity is given by the Wilke-Chang correlation (Wilke and Chang 1955), which is valid (within 10%) for a non-dissociating solute A at low concentrations in a solvent B:

$$D_{A,B}(\mathrm{cm^2 s^{-1}}) = 7.4 \cdot 10^{-8} \frac{(\psi_B \cdot M_B)^{1/2} T}{\mu_B \cdot \tilde{V}_A^{0.6}} \tag{20}$$

where ψ_B is an "association parameter" for the solvent B ($\approx$2.6 for water), M_B is the molar mass of B, T is the absolute temperature, μ_B is the viscosity of B, and $\tilde{V}_A$ is the molar volume of A in $\mathrm{cm^3 g/mole}$ at its normal boiling point.

This is an implicit function of T, since μ_B is also temperature dependent. The relation can be rewritten as

$$D_{A,B}(\mathrm{cm^2 s^{-1}}) = 7.4 \cdot 10^{-8} \cdot \alpha_{A,B} \frac{T}{\mu_B} \tag{21}$$

where $\alpha_{A,B}$ is a constant for the particular system and does not depend on temperature. Conversely, the rest of the equation does not depend on the solute A. Therefore, regardless of the temperature,

$$\sqrt{\left(\frac{D_{H_2,H_2O}}{D_{O_2,H_2O}}\right)} = \sqrt{\left(\frac{\alpha_{H_2,H_2O}}{\alpha_{O_2,H_2O}}\right)} \tag{22}$$

The values of $\alpha_{A,B}$ can be calculated at lower temperatures, where diffusivities and viscosities are both well tabulated (typically between 10°C and 40°C). Using values supplied by the CRC Handbook of Chemistry and Physics (online edition, http://www.hbcpnetbase.com), we find that

$$\sqrt{\left(\frac{\alpha_{H_2,H_2O}}{\alpha_{O_2,H_2O}}\right)} \approx \sqrt{\left(\frac{2.12}{0.913}\right)} = 1.52 \tag{23}$$

We must now make a final correction for temperature. The van't Riet correlation was based on data collected at room temperature, and cannot be directly applied to our system. Using the penetration model once again and assuming that room temperature is roughly 20°C, we obtain

$$k_L a(H_2, 85°C) = 2.0 \cdot 10^{-3} \cdot \left(\frac{W}{V_L}\right)^{0.7} \cdot v_S^{0.2} \cdot \sqrt{\left(\frac{\alpha_{H_2,H_2O}}{\alpha_{O_2,H_2O}}\right)} \cdot \sqrt{\frac{D_{H_2}(85°C)}{D_{H_2}(20°C)}} \tag{24}$$

From the Wilke-Chang correlation we obtain

$$\sqrt{\frac{D_{H_2}(85°C)}{D_{H_2}(20°C)}} = \left(\frac{T(85°C)}{\mu_{H_2O}(85°C)} \cdot \frac{\mu_{H_2O}(20°C)}{T(20°C)}\right)^{\frac{1}{2}} = 1.90 \tag{25}$$

Combining Eqs. 24 and 25 and inserting all parameter values yields

$$k_L a(H_2) \approx 1400\ \mathrm{h^{-1}} \tag{26}$$

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