JOURNAL OF BACTERIOLOGY, Aug. 1978, p. 717-720
0021-9193/78/0135-0717$02.00/0
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Vol. 135, No. 2
Printed in U.S.A.

Occurrence of Squalene in Methanol-Grown Bacteria

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Received for publication 10 March 1978

The nonpolar lipids of methanol-grown bacteria which utilize one-carbon (C_1) compounds via the RMP pathway (*Pseudomonas* C, *Pseudomonas methylotropha*, and *Methylomonas methanolica*) were found to contain squalene in concentrations between 0.1 to 1.16 mg/g of cell (dry weight). Squalene could not be detected in lipid extracts of methanol-grown bacteria which utilize C_1 compounds via the serine pathway.

Squalene is well known as a precursor for steroids (21) and is usually absent in procaryotes (4). In recent years several reports have shown that certain bacteria (2, 8, 11, 17-20) and blue-green algae (cyanobacteria) (7, 14) were capable of synthesizing steroids, and by implication squalene, in a fashion similar to eucaryotic organisms. Although quantitative data are few, they indicate that the amounts of steroids (or squalene) found in procaryotes (0.001 to 0.1 mg/g of cell [dry weight]) are, in most cases, considerable lower than those found in eucaryotic organisms (for example, *Aspergillus nidulans* which contains 0.3 mg of squalene/g of cell [dry weight]) (3, 5). Two striking exceptions are *Halobacterium cutirubrum* (12, 13, 22), in which the amount of squalene is 1 mg/g of cell (dry weight) and *Methylococcus capsulatus* (3, 5), which contains 5.5 mg of squalene/g of cell (dry weight).

M. capsulatus, a methylotroph bacterium, is able to grow on methane or methanol and is similar in several aspects to a large group of bacteria, the methanol utilizers (6, 15). Although many bacteria can grow on methanol but not on methane as a sole source for carbon and energy, the classification of the methanol-utilizing bacteria has still not been resolved (6, 15). One group of these bacteria incorporates the one-carbon (C_1) methyl unit into a pentose phosphate, forming a hexose phosphate (RMP pathway), whereas in the other group the methyl group is added to glycine via transhydroxymethylation forming serine (serine pathway) (6, 15). Another criterion for the classification of methanol-utilizing bacteria was suggested on the basis of the differences in phospholipid and fatty acid composition among the two groups (9).

In this work we have used two groups of methanol-utilizing bacteria: (i) bacteria which utilize C_1 compounds via the RMP pathway—*Pseudomonas* C, *Pseudomonas methylotropha*, and *Methylomonas methanolica* (*Pseudomonas methanolica*, ATCC 21704); and (ii) bacteria which utilize C_1 compounds via the serine pathway—*Pseudomonas* 1, *Pseudomonas* 135, *Pseudomonas* AM-1, and *Pseudomonas* M-27 (9, 10, 16). The results reported here show that the first group of bacteria contains relatively high levels of squalene, and no evidence was found for the presence of squalene in the second group.

Cells were grown in batch cultures in mineral M-3 medium (pH 7.0) (1), supplemented with Na_2HPO_4 (2 g/liter), $Na\ H_2PO_4$ (0.9 g/liter), and methanol (5 to 20 g/liter) at 34°C, as described previously (1). Cells from the late exponential growth phase were harvested by centrifugation and added to an equal volume of acetone. The mixtures were stirred for 2 h at room temperature and extracted eight times with a half volume of benzene. The benzene extracts were combined, dried with anhydrous $MgSO_4$, and concentrated at 32°C. The clear solutions obtained are referred to as "benzene extracts."

A sample from the benzene extract obtained from *Pseudomonas* C was subjected to gas-liquid chromatography (GLC). Figure 1A shows the presence of two minor peaks: (i) having the same retention time as squalene, and (ii) having the same retention time as farnesol. The extract was further purified by thin-layer chromatography (TLC), and the lipid compound obtained gave a single peak (peak 1) when co-chromatographed on GLC with squalene (Fig. 1B) and corresponded exactly to the authentic standard of biosynthetic [^{14}C]squalene when co-chromatographed on a TLC plate (Fig. 2). A second compound was isolated by a similar technique and co-chromatographed by GLC (peak 2) and

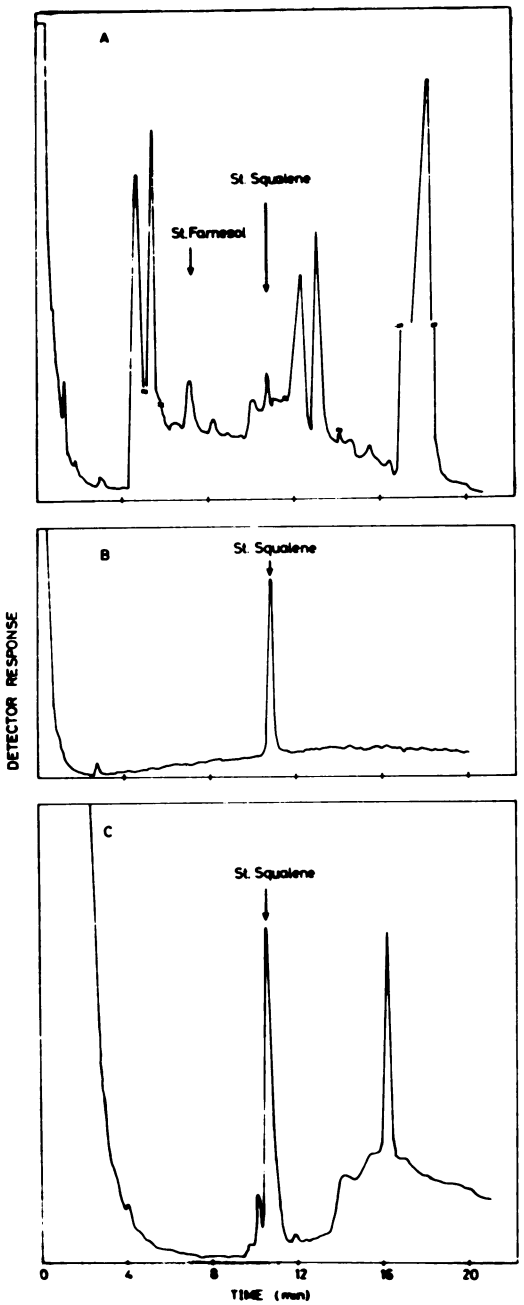

FIG. 1. *GLC of lipids obtained from Pseudomonas C and M. methanolica. (A) A sample from the benzene extract of Pseudomonas C was applied to a Packard model 417 gas chromatograph equipped with a flame ionization detector and a 10% SP 2340 on 100/120 Chromosorb W/AW (Supelco) glass column (0.4 cm by 1.8 m). The injector and detector were at 270°C, whereas the column was programmed from 170 to 250°C at a rate of 6°C/min. (B) A sample from the benzene extract of Pseudomonas C was applied to a pre-coated preparative 2-mm silica gel (F-254, Merck) thin-layer chromatographic plate (4 cm by 20 cm) which was developed to a height of 10 cm with hexane. The area of the TLC plate corresponding to squalene (R_f = 0.52) was scraped and eluted with a solution of 25% ether in hexane. A sample was chromatographed by GLC. (C) A sample from the benzene extract of M. methanolica was chromatographed by GLC as described in (A). Arrows indicate retention of standards.*

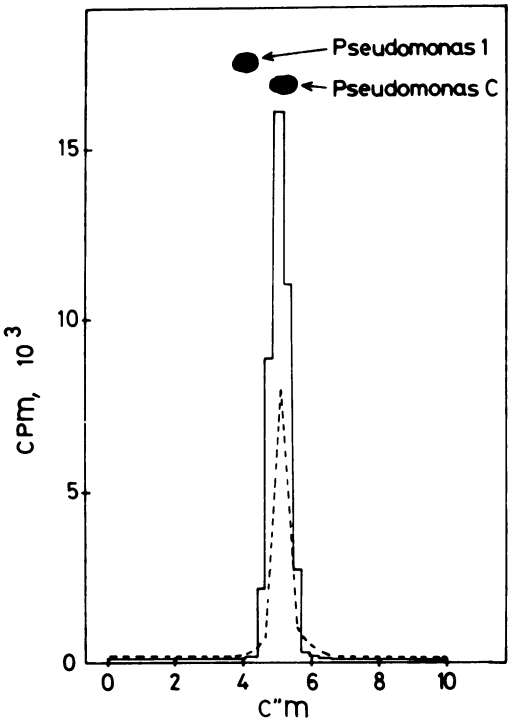

FIG. 2. *Co-chromatography of lipids obtained from Pseudomonas C or Pseudomonas 1 with an authentic standard of biosynthetic all trans [^{14}C]-squalene. [^{14}C]squalene was prepared in an assay mixture containing (in a total volume of 27 ml): phosphate buffer (pH 7.5), 0.1 M; $MgCl_2$, 6 mM; ATP, 6 mM; NADPH, 5 mg; [2-^{14}C]mevalonic acid (22.6 mCi/mmol, New England Nuclear), 50 μCi; and 5 ml of a microsomal suspension prepared from rat liver as described by Yamamoto et al. (23). Incubation was carried out at 37°C for 1 h in nitrogen atmosphere. The reaction was terminated by the addition of an equal volume of acetone, and [^{14}C]squalene was extracted with four 10-ml quantities of hexane. Samples of lipids obtained from Pseudomonas C and Pseudomonas 1 (as described in Fig. 1B and 3B, respectively) were mixed with [^{14}C]squalene and chromatographed on analytical 0.2-mm silica gel TLC plates. Lipids were detected by iodine (dark figures) and analyzed first by scanning for radioactivity (- - -) (Packard model 7200) and then by scraping narrow segments of the gel into vials containing scintillation solution and measuring radioactivity (——).*

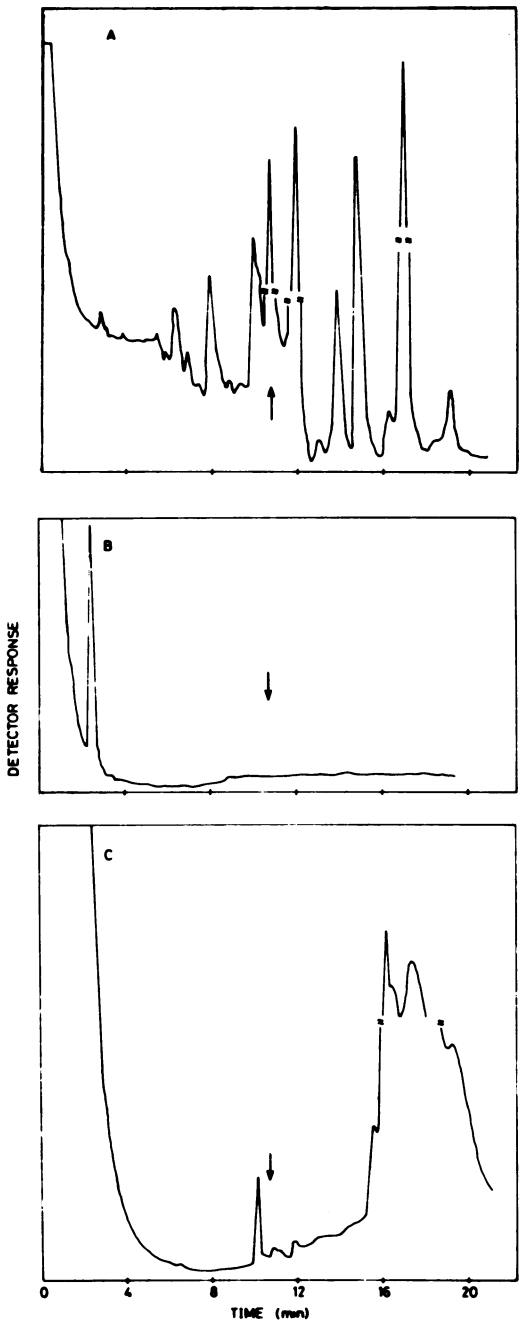

FIG. 3. *GLC of lipids obtained from Pseudomonas 1 and Pseudomonas M-27. (A) A sample from the benzene extract of Pseudomonas 1 was applied to GLC as described in Fig. 1A. (B) A sample from the benzene extract of Pseudomonas 1 was chromatographed on preparative TLC plates. The lipid having a R_f value of about 0.52 was eluted and applied to GLC as described in Fig. 1B. (C) A sample from the benzene extract of Pseudomonas M-27 was applied to GLC as described in Fig. 1A. Arrows indicate retention of standards of squalene.*

TLC with farnesol (data not shown).

Although a generally different GLC pattern was obtained for the benzene extract of *M. methanolica* (Fig. 1C), when the peak having a retention time identical to squalene was isolated it corresponded to standard [^{14}C]squalene when co-chromatographed on a TLC plate (as described for *Pseudomonas* C in Fig. 2), indicating the identity of the compound with squalene. The same results were also obtained with *P. methylotropha* (results not shown).

For the purification of squalene from *Pseudomonas* C, a sample of the benzene extract was evaporated to dryness, dissolved in hexane, and layered onto a column (0.75 cm by 35 cm) of silica gel impregnated with 6% $AgNO_3$ which was pre-equilibrated with hexane. The squalene was eluted from the column with a convex gradient of benzene-ethyl acetate (3:1). Fractions containing squalene were pooled, concentrated under a stream of N_2, and dissolved in a small volume of hexane. After further purification on preparative TLC plates, the compound obtained gave a single peak, when co-chromatographed on GLC with standard squalene (same as Fig. 1B) and corresponded with [^{14}C]squalene upon co-chromatography on a TLC plate (same as Fig. 2).

The presence of squalene was further confirmed by the proton nuclear magnetic resonance spectrum (measured in a Bruker WH-90 Fourier transform spectrometer) of a sample purified from *Pseudomonas* C. The isolated material suspected as squalene has the following characteristics (with the suggested structural features responsible for them in parentheses): a triplet peak at δ = 5.17 ppm (vinyl protons); a peak at δ = 2.02 ppm (allylic protons); two sharp peaks at δ = 1.68 ppm and δ = 1.60 ppm with an area ratio between the two peaks of 1:2.97 accordingly (both are methyl protons, the signal at δ = 1.68 ppm is assigned to a resonance peak of the vinyl *gem* dimethyls, and the signal at δ = 1.60 ppm is assigned to the second resonance peak of the vinyl *gem* dimethyls which overlaps a peak of the vinyl methyls). The area ratios between the vinylic protons, allylic protons, and the methyl protons was found to be 1:3.31:4.1 in agreement with 6, 20, and 24 such protons in squalene. A virtually identical proton nuclear magnetic resonance spectrum was obtained from an authentic standard of squalene.

The amounts of squalene found in RMP bacteria were 0.1, 0.76, and 1.16 mg/g of cell dry weight for *Pseudomonas* C, *P. methylotropha*,

and *M. methanolica*, respectively. The higher values were similar to those found for *H. cutirubrum* (12, 13, 22) and about one-fifth of that measured for *M. capsulatus* (3, 5). In the last bacterium the presence of squalene was accompanied by an extensive membrane system (15). When compared on the basis of the total lipid content (3, 5, 9), *M. capsulatus* contains a higher amount of squalene than the other methanol-utilizing bacteria (55 mg of squalene/g of total lipids as compared to values of 3 to 11 mg of squalene/g of total lipids in methanol utilizers). Another difference between *M. capsulatus* and these methanol utilizers was expressed by the ability of *M. capsulatus* to synthesize zymosterol derivatives from squalene, whereas in the other bacteria no sterols could be detected. (When samples of the benzene extracts of the RMP bacteria were subjected to GLC on a 3% SP 2250 on 100/120 Supelco AW-DMCS column, no sterols could be detected.) As *M. capsulatus* utilizes both methane and methanol via the RMP pathway (6, 15), it was reasonable to suggest that its ability to accumulate relatively large amounts of squalene and to synthesize sterol derivatives was related to methane utilization.

Figure 3A is the GLC pattern of the benzene extract of *Pseudomonas* 1, which utilizes C_1 compounds via the serine pathway, and it shows the presence of a peak having a retention time similar to squalene. However, subsequent purification of the benzene extract of this bacterium followed by GLC (Fig. 3B) and TLC (Fig. 2) showed that squalene could not be detected in this extract. Similar results were obtained for *Pseudomonas* M-27 (Fig. 3C), *Pseudomonas* AM-1, and *Pseudomonas* 135 (data not shown), indicating the absence of significant amounts of squalene in the nonpolar lipid fractions of the serine bacteria.

Although we have shown the presence of relatively high amounts of squalene in bacteria which utilize methanol via the RMP pathway, we have further demonstrated that no detectable amounts were found in bacteria utilizing C_1 compounds via the serine pathway. We, therefore, propose that the presence or the absence of squalene may serve as an additional criterion for the classification of methanol-utilizing bacteria.

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