The Occurrence and Location of Squalene in *Clostridium pasteurianum*

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INTRODUCTION

Squalene, an acyclic triterpene, is the biosynthetic precursor of sterols (Woodward & Bloch, 1953; Eschenmoser *et al.*, 1955) which appear to be important components of animal and plant membranes (Masiak & LeFevre, 1974; Grunwald, 1975). Although the reactions involved in the biosynthesis of squalene proceed under anaerobic conditions (Popják, 1969), the conversion of squalene into sterols *in vivo* involves several oxygen-dependent steps, the first of which is the formation of squalene 2,3-oxide (Corey *et al.*, 1966; Van Tamelin *et al.*, 1966; Yamamoto & Bloch, 1970). Clearly, therefore, anaerobic organisms cannot synthesize sterols but it is possible that they could synthesize squalene. If they were able to do this, then the squalene might be incorporated into their membranes in place of sterol. Until recently, the few reports of the presence of squalene in bacteria had been confined to aerobic species (Suzue *et al.*, 1968; Tornabene *et al.*, 1969; Bird *et al.*, 1971; Bouvier *et al.*, 1976; Goldberg & Shechter, 1978; Tornabene, 1978). However, there are now two reports of squalene being found in some anaerobic bacteria (Amdur *et al.*, 1978; Tornabene *et al.*, 1978). In this paper we report the presence of squalene in the anaerobe *Clostridium pasteurianum*, its concentration in both vegetative and sporulating cultures and its location in the membrane of sporulating cells.

METHODS

Organisms. *Clostridium pasteurianum* strain W-5 (ATCC 6013) was used for all the squalene estimations described except those of mother cell and forespore membranes for which *C. pasteurianum* mutant strain MR505 was used. The latter organism is a granulose-negative mutant of *C. pasteurianum* ATCC 6013 (Robson *et al.*, 1974) from whose sporulating cells forespores are the more easily prepared in the absence of adherent granulose (Clarke, 1978).

Growth and harvesting procedures. The organisms were transferred from single colonies, grown anaerobically at 37 °C on plates of Reinforced Clostridial Medium (RCM; Oxoid CM149) solidified with 1.5 % (w/v) 'lab m' agar, to 20 ml RCM and grown overnight at 37 °C. The resulting culture was used to inoculate 500 ml growth medium and was grown anaerobically overnight at 37 °C. This culture was then used to inoculate 20 l growth medium which was cultured anaerobically at 37 °C. Vegetative cells were harvested by continuous-flow centrifugation (100 to 150 ml min⁻¹) at 18000 *g* after 16 h growth. Sporulating organisms were harvested when microscopic examination of samples of the culture showed that over 80 % of the cells contained phase-dark forespores. The growth medium for *C. pasteurianum* strain W-5 contained (per litre): MgSO₄·7H₂O, 0.1 g; NaCl, 0.1 g; Na₂MoO₄·2H₂O, 0.01 g; ferric sodium EDTA, 0.275 g; CaCl₂·6H₂O, 0.1 g; MnSO₄·4H₂O, 0.015 g; NH₄Cl, 3.0 g; glucose, 20.0 g; biotin, 0.12 mg; *p*-aminobenzoate, 2.0 mg. It was sterilized by autoclaving at 103.4 kPa for 30 min and, after cooling, sterile 1.0 M-KH₂PO₄/KOH buffer, pH 7.0, was added to give a final concentration of 0.05 M. The growth medium for *C. pasteurianum* mutant MR505 was identical to that above except that 1 % (w/v) casein hydrolysate was added in place of NH₄Cl.

The harvested pellet of cells was washed twice by resuspending in 0.1 M-KH₂PO₄/KOH buffer, pH 6.5, containing 6 mM-MgSO₄, and resedimenting by centrifugation. Wet and dry weights of cells were then determined. The washed cells of *C. pasteurianum* strain W-5 were used for determination of squalene content, whilst unwashed cells of *C. pasteurianum* mutant MR505 were converted into protoplasts by treatment with lysozyme prior to the isolation of the mother cell cytosol and of the mother cell, inner forespore and outer forespore membrane fractions (Clarke, 1978). Each of the membrane fractions was washed twice by suspension and centrifugation in 50 mM-KH₂PO₄/KOH buffer, pH 7.0. The squalene content of the cytosol and the three membrane fractions, relative to their protein content, was then determined.

Extraction and analytical procedures. The washed cells of *C. pasteurianum* strain W-5 and the membrane fractions from *C. pasteurianum* mutant MR505 were suspended in 3 vol. ethanol and refluxed with 0.3 vol. 60% (w/v) aqueous KOH solution for 1.5 h in the dark. The saponified mixture was diluted with 4 vol. water and extracted five times with a total of 20 vol. light petroleum (b.p. 40 to 60 °C) which had been purified by drying over sodium-lead alloy, followed by two redistillations and passage down a column of silica gel (BDH, 30 to 120 mesh; 1 l light petroleum per 250 g silica gel). The bulked petroleum extracts were washed with water, dried over anhydrous Na₂SO₄ and evaporated to dryness under oxygen-free nitrogen to give the unsaponifiable lipid. The mother cell cytosol from *C. pasteurianum* mutant MR505 was extracted five times with a total of 5 vol. freshly prepared, dry, peroxide-free diethyl ether. The ethereal extracts were bulked, washed with water, dried over anhydrous Na₂SO₄ and evaporated to dryness under oxygen-free nitrogen to give the lipid.

The unsaponifiable lipid from *C. pasteurianum* strain W-5 was chromatographed on a column of acid-washed alumina (Woelm) weakened with water to Brockman Grade III (10 mg lipid per g alumina), which had been thoroughly washed with purified light petroleum. The column was developed with purified light petroleum (10 ml per g alumina) followed by an equal volume of 2% (v/v) dry, peroxide-free ether in light petroleum. The lipids eluted in these two fractions were subjected to thin-layer chromatography (t.l.c.) on 0.25 mm thick layers of silica gel G impregnated with Rhodamine 6G using light petroleum (b.p. 40 to 60 °C) as solvent. Markers of authentic squalene (R_F 0.2 to 0.4 depending upon the freshness of the plate) were chromatographed on each plate. T.l.c. of the lipids of the 2% ether/light petroleum fraction produced no zone chromatographing near to authentic squalene; these lipids were not investigated further. T.l.c. of the lipids of the light petroleum fraction did, however, produce a zone which co-chromatographed with authentic squalene. This zone was scraped off the plate and extracted with dry, peroxide-free diethyl ether. The unsaponifiable lipids from the three membrane fractions of *C. pasteurianum* mutant MR505 and the lipid from the mother cell cytosol were subjected directly to the above t.l.c. procedure. A zone co-chromatographing with authentic squalene was detected on the chromatographs of the unsaponifiable lipids of all three membrane fractions; these zones were scraped off and extracted as before. No such zone was detected on the chromatogram of the lipid of the mother cell cytosol; nevertheless, that area of the plate where squalene would have been located had it been visible, was scraped off and extracted. The lipid in each of the t.l.c. 'squalene zones' was dissolved in a known volume of spectroscopically pure *n*-hexane and 2 μ l samples were analysed by gas-liquid chromatography (g.l.c.) using a Pye 204 gas chromatograph fitted with a 210 $\times$ 0.4 cm glass column packed with 1% (w/w) SP-2250 (Supelco) on 80 to 100 mesh Supelcoport. The carrier gas was oxygen-free nitrogen (40 ml min⁻¹), the operating temp. was 230 °C and detection was by flame ionization. Authentic squalene had a retention time of about 11 min on this system. Samples of the squalene-containing lipid from t.l.c. were also analysed by mass spectrometry (m.s.) and by g.l.c.-m.s., for which a Pye 104 gas chromatograph was linked via a single-stage silicone rubber membrane separator (Hawes *et al.*, 1969) to an AEI MS-30 mass spectrometer. The column in the gas chromatograph was the same as that described above but the carrier gas was helium (50 ml min⁻¹) and the operating temp. was 220 °C. The ionizing energy used in the m.s. was 24 eV, the emission current was 300 μ A and the source temp. was 220 °C. For m.s. by direct probe injection, the ionizing energy was 70 or 18 eV, the emission current was 100 μ A, the probe temp. was 80 °C and the source temp. was 150 or 180 °C.

Quantitative estimation of squalene. Squalene was estimated from g.l.c. peak areas using a standard curve which related peak area to known weight of authentic squalene.

Protein estimation. Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Analysis of the t.l.c. 'squalene zones' isolated from both vegetative and sporulating cells of *C. pasteurianum* strain W-5 by g.l.c. revealed the presence of a peak which not only had a retention time identical to that of authentic squalene but which co-chromatographed with it. The mass spectrum of the material of this peak showed ions at the following *m/e* values (the

Table 1. *Squalene content of Clostridium pasteurianum and its membranes*

<i>C. pasteurianum</i> strain	State of cells*	Component†	Squalene content	
			$\mu\text{g (g dry wt)}^{-1}\ddagger$	$\mu\text{g (mg protein)}^{-1}$
W-5 (ATCC 6013)	Vegetative	Whole cells	0.88 ± 0.15 (3)	—
	Sporulating	Whole cells	3.41 ± 0.54 (3)	—
MR505	Sporulating	MC cytosol	—	0.003
		MC membrane	—	0.252
		FS outer membrane	—	0.217
		FS inner membrane	—	0.269

* 'Vegetative' indicates that less than 5 % of the cells in the culture had formed forespores; 'sporulating' indicates that more than 80 % of the cells in the culture had formed forespores.

† MC, Mother cell; FS, forespore.

‡ Values are given as means $\pm$ S.E.M.; the number of independent estimates is given in parentheses.

percentage abundance of the ions relative to the main peak and their identification, where known, are given in parentheses): 410 (3.5, M^+); 395 (0.4, $M^+ - \text{Me}$); 367 (1.9, $M^+ - \text{isopropyl}$); 341 (4.7, $M^+ - \text{isoprene}$); 328 (1.0); 299 (1.1); 273 (2.2, $M^+ - 2$ isoprenes); 231 (2.8); 217 (3.0); 205 (3.2, $M^+ - 3$ isoprenes); 203 (6.0); 192 (4.8); 191 (6.0); 189 (5.4); 187 (4.4); 177 (5.9); 175 (6.6); 163 (6.3); 161 (7.8); 159 (5.5); 149 (24.3); 137 (58.3, $M^+ - 4$ isoprenes); 136 (43.4); 135 (26.4); 123 (34.6); 121 (39.9); 109 (37.7); 107 (28.2); 95 (57.0); 93 (38.4); 81 (100); 69 (94.0, $M^+ - 5$ isoprenes). This mass spectrum was identical with that of authentic squalene obtained under the same conditions on the same instrument; it differs from that reported by Tornabene *et al.* (1969) only marginally and even then only in respect of the percentage relative abundances of the ions.

Vegetative cells had an average squalene content of $0.88 \mu\text{g (g dry wt)}^{-1}$ whilst sporulating cells contained about four times as much, with an average content of $3.41 \mu\text{g (g dry wt)}^{-1}$ (Table 1). The major morphological difference between these two types of cell is the presence of forespores in the sporulating cells. The forespore is surrounded by two membranes, an inner and an outer, which are both formed from the mother cell membrane by distinct invagination and fusion processes (Mackey & Morris, 1971). The sporulating cells therefore contained more membrane material than the vegetative cells. It seemed possible that the coincidence of greater squalene content and greater membrane content in the sporulating cells was indicative of a membrane location for squalene in *C. pasteurianum*. Accordingly, the squalene content of the membranes of *C. pasteurianum* was determined and compared with that of the cytosol. These determinations were performed on sporulating cells of *C. pasteurianum* mutant MR505 since it is possible to separate and isolate from such cells the mother cell membrane, the outer forespore membrane and the inner forespore membrane (Clarke, 1978). The squalene content of all three membranes was of the same order, approx. $0.25 \mu\text{g (mg protein)}^{-1}$ (Table 1), probably reflecting their common origin, and was about two orders of magnitude greater than that of the mother cell cytosol. This clearly indicates that squalene is preferentially located in the membranes of *C. pasteurianum*. Whether the squalene is present in these membranes in a quantity sufficient to play a significant role in their function remains to be seen. It is, however, apparent that the squalene content of *C. pasteurianum* is very much lower than that reported in aerobic bacteria (Tornabene *et al.*, 1969; Bird *et al.*, 1971; Bouvier *et al.*, 1976; Goldberg & Shechter, 1978).

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