The Formation of Simple Fruiting Body-like Structures Associated with Sporulation under Aerobic Conditions in *Clostridium acetobutylicum*

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Isolated colonies of *Clostridium acetobutylicum*, grown anaerobically for 2 d before exposure to aerobic conditions, developed into unique elongated fruiting body-like structures which reached a height of >10 mm. The elongated structures were surrounded by an extracellular fibrous substance which formed a tough pliable sheath on exposure to air. Vegetative and sporulating cells were restricted to the basal region of the structure; the trunk region contained mainly free spores and a small number of granulated or lysed cells. Colonies maintained under anaerobic conditions did not produce the elongated macroscopic structures.

**INTRODUCTION**

The only group of bacteria known to produce differentiated fruiting bodies are the myxobacteria (Dworkin, 1966, 1973). Fruiting bodies produced by the myxobacteria vary from simple mounds of cells formed by *Myxococcus* to elaborate lobed forms found in the higher myxobacteria (*Polyangiaceae*). Within the fruiting body the individual vegetative cells lose their gliding motility and become resting cells (myxospores) which in some species are contained within cysts and in others occur as an undifferentiated mass. Although a number of other bacteria do show rudimentary multicellular organization [e.g. *Actinomycetes* (Chater & Hopwood, 1973)] no other group is known to produce fruiting body-like structures.

During a study of bacteriocin production by *Clostridium acetobutylicum* (Barber et al., 1979), we observed the formation of elongated macroscopic structures resembling fruiting bodies. We describe here the production and nature of these structures.

**METHODS**

The *Clostridium acetobutylicum* strain was supplied by National Chemical Products Ltd, Germiston, South Africa. It was grown on the potato medium of Barber *et al.* (1979). Samples (0.5 g) of soil spore cultures of *C. acetobutylicum* were first heat-shocked in 3 ml 0.85% (w/v) NaCl at 70°C for 2 min before being inoculated into potato broth. Cultures were incubated at 34°C for 18 h and plated on potato agar. Duplicate plates were incubated in BBL GasPak jars for a further 48 h. One set of plates was then removed and incubation was continued aerobically at 20°C and the other set was retained under anaerobic conditions at 20°C. Colonies from each set of plates were examined macroscopically and microscopically every 2 d for 28 d. Spore and viability counts were carried out on colonies from both sets of plates. Wet-mounted specimens were observed with a Zeiss photomicroscope fitted with phase contrast optics. Metal-shadowed specimens were examined with a Jeol JSN U3 scanning electron microscope. Photographs of the macroscopic structures were taken with a Pentax SP 500 camera fitted with close-up rings.

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RESULTS

Aerobic fruiting body-like structures

Plates containing isolated colonies of *C. acetobutylicum* were grown under anaerobic conditions for 2 d and then incubated aerobically for up to 4 weeks. Under aerobic conditions the colonies developed into unique elongated macroscopic structures which reached a height of > 10 mm over 3 to 4 weeks (Fig. 1 a, b). These structures only developed in isolated colonies under aerobic conditions and crowding inhibited their development. The final shape of the macroscopic structures depended on the size of the colony at the time of transfer to aerobic conditions. Small colonies produced tall slender structures with a distinct helical twist (Fig. 1 a) and large colonies produced shorter thicker structures.

Dissection and microscopic examination of the elongated structures indicated that they were associated with sporulation. Healthy rod-shaped vegetative cells and sporulating cells were restricted to the basal region of the structure (Fig. 1 e). The trunk region contained mainly free spores (refractile bodies) and no sporulating cells were observed (Fig. 1 f). There were only a few cells in the trunk region and they were either granulated or lysed in appearance.

Vegetative rods and spores within the macroscopic structure were surrounded by an extracellular substance which formed a tough pliable sheath around the colony. The difference between the surface layer (sheath) and the contents of the elongated colony was clearly seen under the scanning electron microscope in colonies which had been fractured during preparation (Fig. 1 c, d). Closely packed vegetative rods near the surface appeared to be covered by a fibrous sheath (Figs 1 d and 2 a).

The difference between the surface of an elongated structure and the surface of an anaerobic colony is shown in Fig. 2(a, b). In the anaerobic colony the individual rod-shaped cells were clearly visible, whereas in the elongated structure the cells were covered by the fibrous sheath. Material from the sheath was dispersed in aqueous solution and observed microscopically. Most of the individual rods appeared to have lysed and were surrounded by an extracellular layer of refractile material (Fig. 1 g).

It was possible to scrape out the soft inner contents of the elongated structure so that only the tough pliable outer sheath remained. After the removal of the inner contents the sheath became hard and brittle. When the surface of an elongated structure was damaged by cutting away the sheath and exposing the inner contents to air, a new tough pliable sheath was rapidly reformed over the damaged area.

Preliminary studies on the chemical composition of the sheath material indicated that it was proteinaceous. It was degraded by the proteolytic enzyme Pronase (Miles-Seravac) but unaffected by cellulyasin (Calbiochem) or lysozyme (Miles-Seravac). It was soluble in sodium dodecyl sulphate but insoluble in water, hot alcohol, chloroform, ether, acetone, butanol, 2 M-HCl or 5 M-NaOH.

A solution of sheath material in sodium dodecyl sulphate absorbed ultraviolet light (280 nm) and could be assayed by the Folin–Ciocalteau test for proteins.

Aerobic development of fruiting body-like structures

The development of the elongated structures was related to the amount of sporulation which occurred within the colony. Sporulation in *C. acetobutylicum* grown on potato agar, under both aerobic and anaerobic conditions, was dependent on the crowding of the colonies on the surface of the plates. The closer the colonies were together, the lower the proportion of cells which sporulated. Crowded colonies did not produce the elongated sporulating structures.

After 48 h growth under anaerobic conditions, isolated colonies were greyish white, very flat with a diffuse margin (Fig. 2 c), and contained only vegetative rods. After 48 h the cells
Fig. 1. Scanning electron micrographs (a to d) and photomicrographs (e to g) showing the morphology and structure of elongated fruiting body-like structures produced by C. acetobutylicum. (a) Elongated structure with a helical twist; (b) development of elongated structures over a 4 week period (right to left); (c, d) fractured elongated structures showing the tough pliable sheath; (e) vegetative rods and sporulating cells from the basal region of the structure; (f) trunk region showing free spores (refractile bodies) and a few cells which were either granulated or lysed in appearance; (g) sheath material dispersed in an aqueous solution showing lysed rods surrounded by extracellular refractile material.
Fig. 2. Scanning electron micrographs (a, b) and photomicrographs (c to f) showing the morphology and structure of aerobic and anaerobic colonies of *C. acetobutylicum*. (a) Surface of an aerobic elongated structure; (b) surface of an anaerobic colony; (c) greyish white flat colonies after 48 h growth under anaerobic conditions (left) and the development of the thicker central sporulating zone after 72 h (right); (d) anaerobic colonies showing the spreading fan of vegetative cells; (e) sporulating cells from the centre of anaerobic colonies (left) and vegetative rods from the fan region of an anaerobic colony (right); (f) spreading fan of an anaerobic colony.
within the isolated colonies began to sporulate under both anaerobic and aerobic conditions. The area of sporulation developed from the centre of aerobic colonies and was easily visible as a thicker and more opaque zone (Fig. 2c). This zone gradually spread outwards, until after 3 to 4 d the entire colony was uniformly thick and opaque. The colony continued to develop vertically and over 14 d the proportion of rods which were healthy in appearance remained constant at about 30% of the total cell population. Sporulating cells accounted for about 60% of the cells. After 14 d the number of rods and sporulating cells decreased, with a concomitant increase in the number of free spores (about 30%) and granulated or lysed rods (about 60%).

In crowded colonies little growth occurred after 48 h. The colonies did not increase in size and remained thin and greyish white. After 6 d about 80% of the cells were granulated or lysed rods, the remainder being vegetative rods and sporulating cells. Only a few free spores were present after 10 d and after 21 d about 10% of the cells were free spores.

Anaerobic colony development

Under anaerobic conditions, isolated colonies produced typical flat spreading colonies which continued to increase in diameter over a period of 28 d. After 14 d, when the colonies were about 5 mm in diameter, large spreading fans grew out from the margins (Fig. 2d, f); these consisted entirely of vegetative rods (Fig. 2e) which contrasted with the actively sporulating cells making up the rest of the colony (Fig. 2e).

DISCUSSION

Under aerobic conditions isolated colonies of the anaerobe C. acetobutylicum produced an elongated structure which contained a high proportion of sporulating cells and free spores enclosed in a tough pliable sheath. This structure seemed to be intermediate between the ordinary bacterial colony and the elaborate multicellular fruiting bodies formed by myxobacteria, and could be regarded as an example of primitive multicellular differentiation.

We have recently examined two other strains of C. acetobutylicum (ATCC 824 and ATCC 10132) but have not as yet observed the production of fruiting body-like structures. However, these strains do not grow as well as our strain on potato agar.

The production under aerobic conditions of an extracellular proteinaceous substance which rapidly hardens on exposure to air to form a sheath is interesting. An obvious suggestion is that it acts as a protective barrier against oxygen. Clostridium acetobutylicum and most other clostridia are fairly strict anaerobes. Free oxygen inhibits growth but C. acetobutylicum can grow in liquid culture in the presence of air provided a sufficiently low oxidation reduction potential is established in the medium (Morris & O'Brien, 1971; O'Brien & Morris, 1971). Clostridium acetobutylicum will not normally grow on solid media under aerobic conditions. Clostridium histolyticum, C. tertium and C. carnis are exceptional in being able to grow aerobically to a limited extent (Wilson & Miles, 1975). Small but visible colonies are formed on blood agar plates but no spores are produced.

Under anaerobic conditions on potato agar, C. acetobutylicum produced flat spreading colonies with fans of vegetative cells. This contrasts with the report of Hastings (1978) who observed that on a molasses agar (under anaerobic conditions) colonies darken slightly and grow in the form of a truncated cone, 2 to 3 mm high, with a concave top. We have only observed the production of the elongated structures, which are not truncated and do not have a concave top, under aerobic conditions.

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REFERENCES


