A Sporulating Strain of *Bacillus popilliae*

Bacillus popilliae was first isolated by DUTKY[1] from diseased larvae of the Japanese beetle, *Popillia japonica* Newm.[1] DUTKY established that the microorganism was the causal agent for the milky disease in the larvae and suggested that the spores of these organisms could be used effectively to control the insect population. Since then many efforts were made to devise a procedure for the cultivation of spores in vitro, especially in liquid media such that the method could be developed to process quantities of spores in large fermentors. Until now, the only successful result obtained in these investigations was the development of a solid medium which allows a sporulation of 0.3%[2]. The majority of these studies were conducted on strains isolated several years ago from larval spores and preserved as lyophilized cultures of vegetative cells. For routine experimentation in the laboratory the strains were maintained by successive transfers of vegetative cells grown in a selective nutrient medium[3]. Such procedures have a certain bias which reflects in the selection of non-sporulating or sparsely sporulating mutants in a population. For, it is a well-known fact that any interruption in the developmental cycle of a culture towards sporulation results in increased loss of viability, especially if only commitment but not completion of sporulation occurs in that medium. Hence we decided to isolate fresh strains capable of sporulation from the spore population obtained from the larvae.

The following method was adopted to isolate strains of *B. popilliae*, bearing in mind a chance observation that these organisms were capable of growth in the presence of chloroform. Lyophilized spores from the larvae were obtained from the Northern Regional Research Laboratories in Peoria, Illinois. The spores were suspended in chloroform and kept at 40 °C for 15 min to increase the percentage of germination. The solvent was removed and the spores were resuspended in 0.01 *M Tris*-HCl buffer at pH 7.0. Different concentrations of spores were spread on agar containing the nutrients outlined in the Table. Several colonies were selected after 48 h incubation at 30 °C and tested for their ability to grow in broth in the presence of chloroform. A liquid medium for optimal sporulation of the isolated strains was developed and its composition is given in the Table.

One of the isolated strains followed closely the morphological changes during vegetative growth and sporulation in the liquid medium as described by DUTKY. Cultures of this strain were grown routinely in 500 ml of YGG medium in a 2-l flask by inoculation of 10^4 spores. The inoculated medium was aerated at 30 °C on a New Brunswick Gyrotory shaker. Samples were taken at intervals and morphological changes were observed under the phase contrast microscope. The organisms showed initial stages of sporulation after 48 h and maximum sporulation was obtained within 72 h. The cultures contained approximately $3–5 \times 10^8$ spores/ml. The spore count was made after heat treatment of the suspension of cells at 80 °C for 15 min. To our knowledge, this is the first instance of sporulating this organism in liquid cultures with yields of nearly 80% sporulation within 72 h.

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Composition of the medium (YGG) for the growth and sporulation of *B. popilliae*

Yeast extract	0.4% (w/v)	Glycerol	0.2% (w/v)
Glucose	0.4% (w/v)	Sodium chloride	0.5% (w/v)

'G' medium contained the following minerals in g/100 ml of distilled water: $(NH_4)_2SO_4$ 0.2; K_2HPO_4 0.05; $MgSO_4$ (anhy.) 0.02; $MnSO_4 \cdot H_2O$ 5×10^{-3}; $CaCl_2$ 8×10^{-3}; $ZnSO_4 \cdot 7H_2O$ 5×10^{-4}; $CuSO_4 \cdot 5H_2O$ 5×10^{-4}; $FeSO_4 \cdot 7H_2O$ 5×10^{-5}.

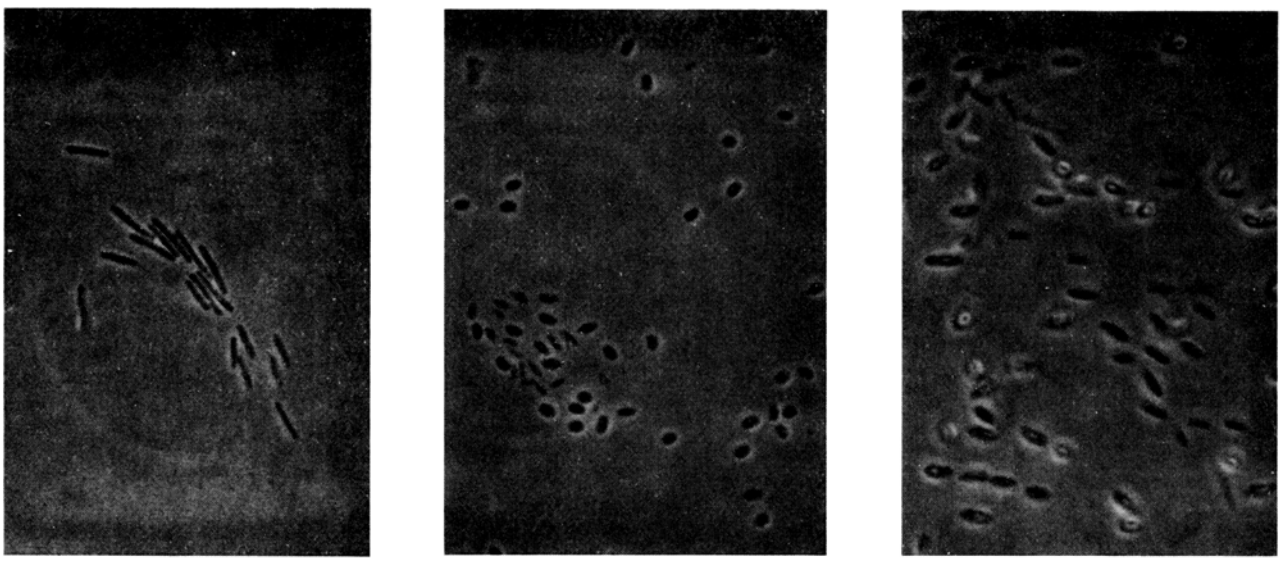

Fig. 1. Phase contrast micrographs of a culture of the sporulating strain of *B. popilliae*. (A) Vegetative cells in the logarithmic phase. × 1200. (B) Early stationary phase. Note the cells are swollen and some of the cells are spindle-shaped. (C) Sporulation phase showing the refractility in the cells.

The organisms are motile, rod-shaped in the vegetative state measuring 4–5 μ in length and 0.6–0.8 μ in diameter. As the transition occurs from vegetative state to commitment to sporulation the cells become swollen and assume a spindle-shape. With the progress of sporulation the refractile body, which appears initially at the terminal end of the organism, moves and is located centrally in the mature spore (Figure 1). The electron micrograph shown in Figure 2 of a thin section of a refractile body bears cytological evidence to the fact that the refractility is indeed due to spore formation and not to the accumulation of lipoid material[3]. In the cells containing the immature spore there can be noticed a granular area corresponding to the position where the parasporal inclusion usually develops. When the spore matures, however, the parasporal body is not intact inside the sporangium but released in the medium. During that time an active proteolytic enzyme is secreted in the medium. Preliminary investigations have shown that the enzyme exhibits differences in properties from those that have been isolated during the sporulation of *B. subtilis* and *B. licheniformis*[4,5]. It is interesting to speculate whether the parasporal inclusion is indeed a pro-enzyme which is activated under conditions of sporulation in vitro and secreted as a proteolytic enzyme[6].

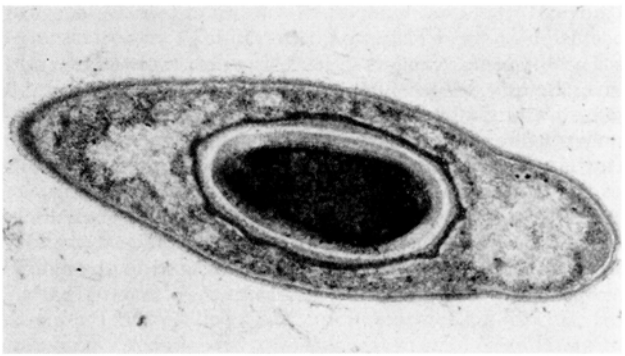

Fig. 2. Electron micrograph of a refractile spore intact within the sporangium. Spores were fixed in osmium tetroxide and counterstained with lead hydroxide after sectioning. Pictures were taken at a magnification × 27,000 and enlarged.

Zusammenfassung. Durch Behandlung mit Chloroform ist es möglich, *Bacillus popilliae* in flüssigem Medium zur Sporenbildung zu veranlassen. Diese Bakterien können zur Bekämpfung von Insekten, der *Popilliae japonica*, benützt werden.

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[6] Our grateful thanks are due to Mrs. Joanne Parker who helped us with the electron micrograph.

STUDIORUM PROGRESSUS

Lymphatic Drainage of the Brain

Investigators studying the pathomechanism of cerebral edema are aware of the difficulties inherent in the production of an experimental edema of the brain. Experimental brain tumours, experimental brain injuries, or freezing of the brain surface, and recently administration of triethyl tin, are common methods, though admittedly rough procedures that finally result in various degrees of edema.

Obviously, nobody has tried to produce a cerebral edema by obstructing lymph flow of the brain, since a well-known fact for first-grade students of medicine is that the brain lacks lymphatics. In this paper, the strange fact will be discussed that, unexpected as it is, by means of obstruction of the cervical lymphatic pathways, a massive cerebral edema can be produced with great regularity in various experimental animals, characterized by gross anatomical, histological, functional and clinical signs.

Connections between the CNS and the lymphatic system. 'With regard to the lymphatic pathways, it is generally agreed that there are no lymphatics within the CNS or meninges'[1].

On the other hand, the presence of lymphatics in the nasal cavity, in the orbita and around the jugular foramen, in the leptomeningeal sheaths around cranial nerves is well known. Different tracer substances injected intrathecally regularly appear in the cervical lymph glands. Nevertheless, no functional significance is ascribed to this lymphatic drainage in the fluid exchange of intracranial structures, since, in the experiments of Courtice and Simmonds[1], the rate of absorption of labelled protein and erythrocytes was not affected by ligation of the main cervical lymphatics.

The above experimental results appear to be supported also by sophistication: (a) according to the present view, the main role of lymphatics is the re-transport of proteins escaped from blood capillaries from the tissues into the blood stream; (b) brain tissue is vigorously protected from the entrance of plasma proteins by the blood-brain-barrier (BBB); hence, there is no need of a lymphatic system; (c) in full accordance, neither classical morphology nor electron microscopy reveals any lymph vessels in the brain; their absence was repeatedly confirmed in the course of our own studies also.

Recent studies, however, revealed that the protection of the brain by the BBB is not an absolute one: (a) a very limited but by no means negligible amount of plasma protein molecules is steadily escaping the blood capillaries and entering the brain substance[2]; (b) several areas of

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