SEQUENCE OF STRUCTURAL CHANGES IN CULTURES OF *CLOSTRIDIUM TETANI* GROWN ON A SOLID MEDIUM

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PLATES XX-XXV

THE majority of *Clostridium tetani* strains will gradually completely spread as a very delicate film across the surface of a solid medium such as blood agar. This phenomenon of swarming growth is of diagnostic importance (Willis, 1964; Willis and Williams, 1970) and can also be made use of in the isolation of *C. tetani* from a mixture with other bacteria (Fildes, 1925). Swarming is demonstrated by other clostridia, for example *C. novyi* (Turner and Eales, 1941) and *C. septicum* (Willis, 1964; Willis and Williams, 1972); by some species of *Bacillus* (Sasarman, Ganea and Dospina, 1957) such as *B. alvei* (Henrichsen, 1972); and by *Proteus vulgaris* and *P. mirabilis* (see Hoeniger, 1964, 1965, 1966, for reviews of the literature).

The present paper describes the sequence of structural changes observed in a strain of *C. tetani* during swarming growth. Cell morphology was studied by phase-contrast microscopy of agar-block preparations and by electron microscopy of negatively-stained preparations of whole organisms. We have also studied the distribution of cellular components such as nuclei and cytoplasm in swarmers stained for ordinary light microscopy or in similar cells embedded and sectioned for electron microscopy. Such a detailed investigation of *C. tetani* has not previously been carried out although the cytology of swarming of this bacterium was briefly discussed by Henrichsen (1972) in a general review of bacterial surface translocation.

MATERIALS AND METHODS

Organism, culture media and procedures

The organism used was *C. tetani* NCTC9575. Stock cultures were grown for 3 days in Bacto-Cooked Meat Medium (Difco) at 37°C, and then held at room temperature for 10 days to induce sporulation (Takagi, Nakamura and Ueda, 1965); glycerol was added to a final concentration of 10% and 1–2 ml samples of the glycerinated culture were stored at –10°C. For use, a glycerinated culture was thawed and inoculated into TPGY broth (described below) and grown overnight at 37°C. Cells from TPGY broth were spread according to the appropriate method on TPGY agar; for phase-contrast studies 1 ml of this medium was poured into a 10-cm glass Petri dish already containing a sterile glass slide and then dried for 5–10 min. at 37°C; when cells were to be stained for their nuclei or cytoplasm, 15 ml of the agar in a dish was dried for 20–25 min.

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TPGY broth (Eklund, Poysky and Boatman, 1969) contains trypticase, 5%; peptone, 0.5%; glucose, 0.1%; yeast extract, 0.5%; and sodium thioglycollate, 0.1%; final pH 7.1. TPGY agar was prepared with 2.0% Noble (Difco) agar. For de-aeration, liquid media were boiled for 15 min., then cooled. Agar plates were incubated in an anaerobic jar (Baird and Tatlock, London) under hydrogen at 37°C.

To study changes with phase-contrast microscopy during the first 6 hours of growth, the inoculum was spread with a 5-mm loop in a stroke overlying the slide cast into the agar; for observations after incubation for 24, 48 and 72 hours, the inoculum was spread to a width of about 1 cm at one side of the dish and at right angles to the length of the slide.

Studies with light microscopy

For observation of "living" cells with the phase-contrast microscope, blocks of agar (approximately 10 mm square and 1 mm deep) were excised from above the immersed slide, mounted on a second slide, cell-side upwards, covered carefully with a no. 0 grease-free glass slip, and sealed on two sides with wax from a small candle.

To stain either the nuclei or cytoplasm, blocks of agar were cut at the edge of swarming with an iridectomy knife, and inverted on to grease-free no. 1 cover-glasses to make impression prints. In preparation for nuclear staining by the osmium-HCl-Giemsa method (Robinow, 1942 and 1944), the cover-glasses bore a film of dried 8% egg white to prevent coalescence of the cells. Further, the staining time with Giemsa was prolonged to 2 hours at 37°C and the preparations were counter-stained for 1 min. in 0.01% basic fuchsin prepared in phosphate buffer, pH 6.8. The cytoplasm was stained with 0.01% thionin for 7.5 min., after the bacteria had been fixed *in situ* through the agar with Bouin's fluid for 1 hour (Robinow, 1944).

Preparations were photographed through a Zeiss Universal microscope equipped with Optovar (set at 1.25), 8× compensating ocular, an achromatic aplanatic condenser (VZ) of N.A. 1.4, and either a neofluor phase-contrast 100× objective with N.A. 1.3 or a planachromatic 100× objective having N.A. 1.32. Light from a tungsten filament lamp was adjusted to give Koehler illumination. It was passed through a broad-band green interference filter (Schott, 546 nm) for phase-contrast examination; and through either a green glass filter (Zeiss VG 9) in combination with an orange gelatin filter (Wratten No. 22) to increase contrast in the nuclei, or through a narrow-band green interference filter (Schott 546±5 nm) for cytoplasm.

Studies with electron microscopy

For *thin sectioning*, swarmers were fixed with osmium tetroxide according to the method of Ryter and Kellenberger (R-K; Ryter and Kellenberger, 1958), post-fixed with 0.5% uranyl acetate, dehydrated in a graded series of alcohols, passed through propylene oxide, followed by propylene oxide plus Epon 812 (1:1), and embedded in Epon. Sections were cut on a Porter-Blum ultramicrotome MT-2 with a diamond knife. They were doubly stained with 2.0% uranyl acetate for 20 min. followed by lead citrate for 10 min.

For *negative staining*, overnight broth cultures (inoculum) were washed and resuspended in distilled water at pH 7.0; cells removed from appropriate regions of plate cultures were likewise taken up in distilled water, pH 7.0. One part of the bacterial suspension was then mixed with one part of a mixture of 2.0% sodium phosphotungstate (Na-PTA, pH 7.1) and 0.05% bovine serum albumin (BSA), pH 7.0 (Hoeniger, 1965). A drop of the preparation was placed on a grid covered with a Formvar film reinforced with carbon, and excess fluid removed after 15 s with a wick of filter paper.

Micrographs were taken with a Philips 300 electron microscope operating at 60 kV.

RESULTS

Observations by phase-contrast microscopy

The sequence of changes on an agar medium could be followed by excising blocks from plates that had been incubated for varying periods of time, i.e., at

STRUCTURAL CHANGES IN *Clostridium tetani*

FIGS. 1-4.—Sequence of changes in an agar slide culture of *C. tetani* excised after incubation for 2, 3, 4 and 6 hours. Phase-contrast microscopy. $\times 2400$.

STRUCTURAL CHANGES IN *Clostridium tetani*

FIGS. 5 and 6.—Edge of growth in an agar slide culture after incubation for 24 and 48 hours. Phase-contrast microscopy. $\times 2400$.

FIG. 7.—Cells bearing forespores or mature spores interspersed with lysing bacteria. Agar slide culture excised from a 72-hour plate. Phase-contrast microscopy. $\times 2400$.

STRUCTURAL CHANGES IN *Clostridium tetani*

FIG. 8.—Nuclei in swimmers. Osmium-HCl-Giemsa technique. Ordinary light microscopy. $\times 2000$

FIG. 9.—Staining of cytoplasmic bands in swimmers by Bouin-thionin procedure. CY, cytoplasm; N, nucleus. Ordinary light microscopy. $\times 2100$.

FIG. 10.—Electron micrograph of longitudinal thin section of swimmer after Ryter-Kellenberger fixation. N, nucleoplasm; C, capsular material; CW, cell wall; PM, plasma membrane. $\times 17,000$.

STRUCTURAL CHANGES IN *Clostridium tetani*

FIG. 11.—Electron micrograph of inoculum cells from overnight broth culture. Negatively stained with Na-PTA/BSA. $\times 9000$.

FIG. 12.—Negatively-stained swimmers from the edge of growth on an overnight plate. Na-PTA/BSA. $\times 9000$.

STRUCTURAL CHANGES IN *Clostridium tetani*

FIG. 13.—Negatively-stained swarmers from the edge of growth on an overnight plate. Na-PTA/BSA. $\times 9500$.

STRUCTURAL CHANGES IN *Clostridium tetani*

FIG. 14.—Short cell from a 48-hour plate culture. Negatively stained with Na-PTA/BSA. $\times 11,000$

FIG. 15.—Rod with terminal forespore taken from a 72-hour culture. Negatively stained with Na-PTA/BSA. $\times 11,000$.

intervals up to 6 hours, and at 24, 48 and 72 hours. Because *C. tetani* is a strict anaerobe it was impossible to follow the sequence continuously in a single preparation as was previously done with *P. mirabilis* (Hoeniger, 1964).

When blocks were excised after incubation for 2 hours a mixture of viable (dark) and non-viable (irregularly mottled grey) cells was observed (fig. 1). This mottling is distinct from the regularly alternating light and dark areas in viable cells seen later (figs. 3 and 4). Nor should it be confused with the optical effect observed in phase-contrast preparations where closely packed groups of bacteria appear darker at the periphery than in the centre. An hour later, the cells had elongated significantly from their original length of 3–5 μm , as can be seen in fig. 2 of a scattered group of viable organisms. The bacteria continued to lengthen and also to multiply so that by 4 hours (fig. 3) small groups of swimmers had formed. These swimmers measured about 5–20 μm in length and showed regular bands of light material, known from the work of Mason and Powelson (1956) and others to represent the sites of nuclear material, separated by dark bands of cytoplasm. By 6 hours (fig. 4) larger groups of swimmers had formed, some individuals being about 30 μm in length.

Swimmers continued to develop, and plates incubated overnight showed a delicate film having an extremely wavy edge. Within the same batch of plates, the film extended varying distances from the site of lateral inoculation; in some cases after incubation for 24 hours the swimmers had moved about half-way across the agar; in others, they had almost covered the plate. Fig. 5 shows a typical group of organisms from the edge of growth. When cells were removed from this region and dispersed in TPGY broth, their length as determined by phase-contrast was found to vary widely, i.e., 19 ± 25.8 μm (mean $\pm$ SD). These data are based on measurements of more than 200 swimmers. The swimmers continued to migrate across the agar plate until the surface was covered. Then, by 48 hours (fig. 6), many of them divided to form shorter cells that either died (light grey organisms in fig. 6) or subsequently sporulated. The latter process was evident in 72-hour cultures (fig. 7), some cells having formed dark forespores, others mature refractile spores. In between the sporulating bacteria in this figure lysing organisms can be seen. By comparing figs. 5 and 7, the difference between the highly variable length of the swimmers and the regular size of the sporing rods derived from them is evident. This suggests a change in the regulation of cell growth and division.

Ordinary light and electron microscopy of fixed swimmers

When stained for their chromatin, swimmers excised on blocks of agar at the edge of the delicate film produced after overnight growth on plates were found to contain a series of single or paired nuclei neatly distributed along their entire length (fig. 8). Measurements of about 50 swimmers in Giemsa-stained preparations showed that the average spacing of the nuclei was 1.546 ± 0.241 μm (mean $\pm$ SD); in calculating these data it was not possible to differentiate between swimmers with single nuclei and those with paired nuclei since both arrangements often occurred in the same organism. That the nuclei are

separated by solid bands of cytoplasm is shown in the Bouin-thionin preparation of fig. 9. In this figure, the cytoplasm (CY) stains darkly while the nuclei (N) appear grey and have a soft contour similar to that observed in phase-contrast micrographs (figs. 3 and 4).

A search for septation by suitable methods of staining for light microscopy gave equivocal results. Therefore, swarmers were embedded for electron microscopy of thin sections. Observations carried out on longitudinal sections made clear that the nuclei are evenly dispersed within the cytoplasm and that there are no intervening cross-septa. The R-K-fixed swarmer in fig. 10 is *c.* 10 μm in length and possesses five regions of fibrillar nucleoplasm (N) several of which are in the process of division. Finely dispersed capsular material (C) surrounds the organisms; the peripheral cell wall (CW) is relatively thin and there is a well-defined plasma membrane (PM).

TABLE
Change in flagellar complement with age of culture on solid medium

Period of culture on solid medium (hours)	No. flagella/unit vol. bacterium (μm^3)		Total flagellar length (μm) /unit vol. bacterium (μm^3)	
	Mean	SD	Mean	SD
0*	8.100	2.897	24.092	6.570
24	37.784	8.477	241.522	53.517
48	18.992	4.353	117.392	28.560
72	1.931	...	7.127	...

* 0 = Inoculum examined.

Electronmicroscopy of negatively-stained bacteria

The sequence of change in flagellar complement accompanying the differentiation of swarmers, their subsequent breakdown into short cells, and spore formation were observed by negatively staining inoculum cells from an overnight broth culture and cells removed from agar plates after incubation for 24, 48 and 72 hours. The inoculum cells in fig. 11 are *c.* 3–5 μm long and bear an average number of 18 flagella of variable length ($3.622 \pm 1.696 \mu\text{m}$). Cells taken from the film of swarming on a 24-hour plate may be relatively short (as in fig. 12) or long; many of the long swarmers are already dividing, as in fig. 13. A notable feature of both figs. 12 and 13 is the significant increase in the number of flagella per unit length. The long swarmer (5 μm) in fig. 12 bears *c.* 150 flagella, while the longer dividing organism in fig. 13 is surrounded by a mass of *c.* 400 flagella. As noted by phase-contrast microscopy (fig. 6), after the swarmers have spread over the agar they break down into short cells. Such a short cell from a 48-hour plate is shown in fig. 14: it is 3 μm in length and bears *c.* 60 flagella $6.142 \pm 1.154 \mu\text{m}$ long. With the onset of sporulation (fig. 15), flagella are no longer synthesised: the rod with terminal forespore taken from a 72-hour plate is surrounded by eight sparse flagella of variable length. The best

measure of the increase and decrease in flagellar synthesis in a plate culture of *C. tetani* is given by examination of electron micrographs and calculating the number of flagella per unit volume of bacterium and the total flagellar length per unit volume. The data are presented in the table: measurements of inoculum cells, 24-hour cells and 48-hour cells were determined from 5–10 micrographs; data for 72-hour cells are based on measurements taken from a single micrograph (fig. 15).

DISCUSSION

Although several earlier investigations had revealed the tendency of *C. tetani* to grow as a spreading film on a solid medium, it was Fildes (1925) who first examined the phenomenon in detail and employed it to isolate 75 new strains from about 500 specimens. This spreading growth of swarming is exhibited by most strains of *C. tetani*. During the present study we found that nine out of ten strains swarmed on sheep blood agar or TPGY agar.

Recently, Willis and Williams (1970) and Williams (1971) have inhibited the swarming of *C. tetani* by incorporating into the medium commercial tetanus antitoxic serum. The inhibition they believe to be due to the presence in the antiserum of agglutinating O antibody to the vegetative cell. Further, these authors have employed such plates for making surface viable counts of *C. tetani* (Williams and Willis, 1970).

The swarming of *C. tetani* can be compared at both the macroscopic and microscopic level with that of *P. mirabilis* which has been studied in detail (Hoeniger, 1964, 1965, 1966). It is well known that the swarming of *C. tetani* is a continuous process during which a delicate but irregularly-edged film of long organisms ('swarmers') moves gradually across the agar surface (fig. 7). On the other hand, the swarming of *Proteus* is a cyclic phenomenon in which there is a regular alteration between a period of swarming and one of consolidation when short cells multiply (see Hoeniger, 1964, for appropriate references). This results in the characteristic sequence of rings seen on a centrally inoculated plate (see Hoeniger, 1964, fig. 1).

At the microscopic level, both *P. mirabilis* and *C. tetani* develop from short cells to swarmers by an inhibition of cell division. These swarmers frequently occur in rafts or groups when viewed with phase-contrast (figs. 3 and 4). After overnight incubation, in the case of *C. tetani*, the rafts of swarmers remain somewhat isolated along the periphery of growth and the individual cells may also appear separate (fig. 5); on the other hand, with *P. mirabilis* the undulating edge of swarming consists of confluent swirls of cells (Hoeniger, 1964).

In the present study, cells located towards the centre of the groups in figs. 3 and 4 show light areas of nucleoplasm and dark bands of cytoplasm, whereas peripheral swarmers appear uniformly dark. This is a general observation with groups of bacteria viewed by phase-contrast and it may be that there is a higher refractive index at the centre than at the periphery. The appearance of the inner cells resembles that of bacteria mounted in a medium of high refractive index such as one containing 15–35% gelatin (Mason and Powelson, 1956) so as to reveal their internal structure.

In the case of stained preparations of both *C. tetani* (figs. 8 and 9) and *P. mirabilis* (figs. 10 and 12 in the paper of Hoeniger, 1966) the nuclei are seen to be evenly distributed along the length of the swarmer and separated from one another by bands of cytoplasm. However, thin sections of *C. tetani* swarmers (fig. 10) failed to demonstrate intervening cross-septa, a feature also absent from *P. mirabilis* (figs. 9–11 in van Iterson, Hoeniger and van Zanten, 1966). Failing demonstration of such cross-septa one may have to look for some mechanism operating at the level of the plasma membrane to explain the neat separation of the nuclear structures within the cytoplasm.

It is apparent from fig. 8 that during the differentiation of *C. tetani* swarmers, DNA replication continues while cell division is inhibited. This merits consideration in relation to the model of the cell cycle in *Escherichia coli* recently put forward by Donachie and his colleagues (Donachie, 1973; Donachie, Jones and Teather, 1973). According to these workers, two parallel and separate sequences of events are initiated within the cell at each doubling: first, DNA replication which is followed by the synthesis of a specific "termination protein"; and second, protein synthesis which is followed by the assembly of some septum precursor(s). Interaction between "termination protein" and septum precursor(s) then leads to cell division. In swarmers of *C. tetani*, as well as in those of *Proteus* (Hoeniger, 1966), it is possible that neither "termination protein" nor septum precursor(s) are synthesised; thus long cells are formed containing numerous and regularly distributed sites of DNA. With regard to *Proteus* swarmers, it had already been shown (Hoeniger, 1966) that they are quite different from the long forms ("snakes", filaments) that arise in response to such treatments as exposure to low concentrations of penicillin or to low dosages of ultraviolet and of gamma irradiation. In the latter forms, the nuclear material assumes either a reticulate or contracted rope-like appearance which points to an inhibitory effect on the stage of DNA replication in the cell cycle.

An outstanding feature of the differentiation of swarmers of both *C. tetani* and *P. mirabilis* is the synthesis of large numbers of flagella. Following the peak of synthesis in both organisms there is a decrease as the swarmers break down into short cells; in the case of *C. tetani* many of the latter then sporulate.

The long filamentous appearance of swarmers of *C. tetani* has been demonstrated in smears stained with methylene blue (fig. 10 of Fildes, 1925), in films stained for flagella (fig. 4 insert of Henrichsen, 1972), in preparations stained with fluorescent labelled antibody (fig. 6 of Batty and Walker, 1963; fig. 3 of Batty and Walker, 1964), and by low power phase-contrast microscopy of growth on the surface of an agar plate (fig. 4 of Henrichsen, 1972). One of the most interesting previous investigations is that of Neumann (1930) who followed continuously with the light microscope the sequence commencing with the germination of a single spore of *C. tetani* in a thin layer of agar in which a colony of *Serratia marcescens* was grown near the field of view, i.e., by adapting to the microscopic level the Fortner anaerobic technique (Fortner, 1929). The original germling was observed to divide several times forming short rods that then developed into a mass of long filaments or "Schwärmern" (figs. 18–20 of Neumann, 1930). No satisfactory explanation has yet been given for swarming

although various suggestions for *Proteus* swarming have been made including the accumulation of a toxic "swarming factor" in the medium (Hughes, 1957) and a nutritional deficiency (see Hoeniger, 1964; Brogan, Nettleton and Reid, 1971; Grabow, 1972). Recently, Grabow (1972) showed that *P. mirabilis* and *P. vulgaris* excrete growth-inhibiting metabolites that are dialysable, volatile and inactivated by ether and chloroform; he thus supports the former view. On the other hand, Brogan *et al.* (1971) observed that the addition of a glycopeptide fraction of yeast extract to a semi-synthetic medium promotes swarming of *P. mirabilis*; this promotion was enhanced by the addition of vitamins of the B group. Perhaps because of the difficulties inherent in the strictly anaerobic nature of *C. tetani*, no comparable experiments into the factor(s) involved in its swarming have been carried out. If the isolation procedure exploited by Fildes (1925) is of practical value, a knowledge of these factors would be of more than academic interest.

SUMMARY

The life cycle of a swarming culture of *Clostridium tetani* has been examined by light and electron microscopy. Onset of swarming is accompanied by cell elongation and nuclear multiplication without cellular division. As swarming progresses, the elongating bacteria produce a greatly increased complement of flagella; the nuclei are seen to be neatly and evenly distributed throughout the cytoplasm and not separated by cross-septa. These findings are discussed in the light of recent studies on the swarming of *Proteus* species.

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