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EXPERIMENTAL ARTICLES

Mechanisms of Forespore Formation during Polysporogenesis in the Anaerobic Bacterium *Anaerobacter polyendosporus* PS-1^T

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Abstract—Forespore formation in the anaerobic bacterium *Anaerobacter polyendosporus* PS-1^T was studied by phase contrast, fluorescence, and electron microscopy. It is concluded that in this bacterium the formation of all forespores in multispore sporangia occurs via the same mechanism as that operating in all known bacilli and clostridia during the single-spore variant of endogenous sporogenesis. Its cytological indicators are as follows: (1) formation of the forespore septum, (2) engulfment of the smaller prespore cell by the larger mother cell, (3) cortex synthesis, (4) assembly of the spore coats, (5) exosporium formation, and (6) lysis of the mother cell. Polysporogenesis in strain PS-1^T is characterized by synchronous formation of all spores (siblings) in a given sporangium and by the absence of any indication of forespore division within the mother cell. These data suggest that multiple spores within a single PS-1^T cell result not from division of the first forespores developing at one or two cell poles, as it was reported for another polysporogenic bacterium, *"Metabacterium polyspora"*, but rather from simultaneous independent formation of several prespores in a single mother cell in the course of modified cell division.

Keywords: cell polarity, polarity transversion, polysporogenesis, ultrastructural organization, nucleoid, prespores, forespores, sibling spores

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The single-spore variant of endogenous sporogenesis has been thoroughly studied in *Bacillus* species by various methods of cytology, physiology, biochemistry, and molecular genetics. The fundamental regularities of this unique biological process have been revealed, making it possible to elucidate the events leading to the emergence of a new, young cell within an old (mother) cell, as well as the peculiarities of cell differentiation in prokaryotes and of de novo formation of unique cell structures (Fitz-James and Young, 1969; Long et al., 1983; Zupancic et al., 2001; Errington, 1993, 2003; Iber et al., 2006; Duda, 1982). The most important conclusion derived from these researches is that endogenous sporogenesis is a special case of modified cell division. In this respect, the investigation of bacterial polysporogenesis opens up new prospects for studying structural and functional capacities of prokaryotic cells. The subjects of such studies may be such multispore bacteria as the cultivated strain of the anaerobic soil bacterium *Anaerobacter polyendosporus* and *"Metabacterium polyspora,"* an uncultivated inhabitant of guinea pig cecum. The endospores of *A. polyendosporus* have characteristics typical of bacterial endospores (ES): they contain dipicolinic acid, demonstrate enhanced heat resistance, and possess the structures typical of ES (Duda et al., 1987; Siunov et al., 1999). Recently we have shown that the determining factor for endogenous polysporogenesis implementation is the process of cell transversion from bipolarity to multipolarity, which is accompanied by the formation of numerous peripherally located nucleoids bound to the cytoplasmic membrane. These nucleoids become the centers of ES formation (Duda et al., 2014). However, the subsequent cytological processes of polysporogenesis have not been studied in detail. Meanwhile, these data are important for understanding the events leading to ES formation in multispore sporangia. Based on the studies of "*Metabacterium polyspora,*" Angert and Losick (1998) and Ward and Angert (2008) arrived at the conclusion that the formation of numerous ES in a single sporangium results from multiplication of the first two forespores originally emerging at the two geometric poles of the rod-shaped mother cell. The possibility of implementation of this polysporogenesis scenario in *A. polyendosporus* has not been investigated. Therefore, the present study was aimed at a detailed cytological investigation of the sequence of processes that occur during sporogenesis in the bacterium *A. polyendosporus* PS-1^T.

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MATERIALS AND METHODS

Objects of study. The strain used in this work was *A. polyendosporus* PS-1^T, described in detail in the papers by Duda et al. (1987) and Siunov et al. (1999). The bacterium was grown on the following media: potato agar and liquid or agarized synthetic medium with the mineral element composition proposed by Pfennig (1965). The synthetic medium was supplemented with trace elements according to Pfennig and Lippert (1966); yeast extract (Difco), 0.05%; glucose or galactose at different concentrations: 0.1, 0.2, 0.3, 0.5, 1.0, 1.5, or 2%; and sodium thioglycollate, 0.1 mg/mL. The cultures in the liquid medium were grown using the Hungate (1966) technique, while those grown on agarized medium were incubated in anaerobic jars filled with oxygen-free N_2 (95%) and CO_2 (5%).

Phase contrast microscopy. Specimens were studied by phase contrast microscopy using LUMAM (LOMO, Russia) and OPTON ICM 405 (Zeiss, Germany) light microscopes.

Fluorescence microscopy. For epifluorescence microscopy, the cells were fixed with 1.5% glutaraldehyde for 30 min and stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Inc.) at a concentration of 1 μg/mL for 5 min. Intravital fluorochromation with acridine orange (AO) (5 μg/mL) was performed at pH 6.0 for 5 min. The specimens were examined under LUMAM (LOMO, Russia) and Polyvar (Reichert, Austria) microscopes; the fluorescence was induced with UV light (maximum at 360 nm) for the specimens fluorochromed with DAPI and with blue light (a 400–490 nm filter) for the specimens stained with AO. The application of AO is important because it provides intravital staining. Since the bacterium under study has no cytoplasmic inclusions, green fluorescent structures can be referred to nucleoids, while red or orange fluorescence under these conditions is a property of forespores.

Electron microscopy of ultrathin sections. Ultrathin sections were prepared as follows. Cells were concentrated by centrifugation (10000 *g*, 15 min), fixed with a 2.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) for 1 h at 4°C, washed three times with 0.05 M cacodylate buffer (pH 7.2), and additionally fixed with a 2% OsO_4 solution in the same buffer for 4 h at 18–20°C. The material was placed into agar, dehydrated with ethanol, and embedded into Epon 812. The capsules with the material were kept in a thermostat at 37°C for 24 h and then at 60°C for 48 h. Upon completion of polymerization, ultrathin sections were made with an LKB 2128 Ultratome (LKB Produkter, Sweden). The sections were mounted on formvar-coated grids, stained with a 3% uranyl acetate solution in 70% ethanol, and additionally contrasted with lead citrate according to Reynolds (1963).

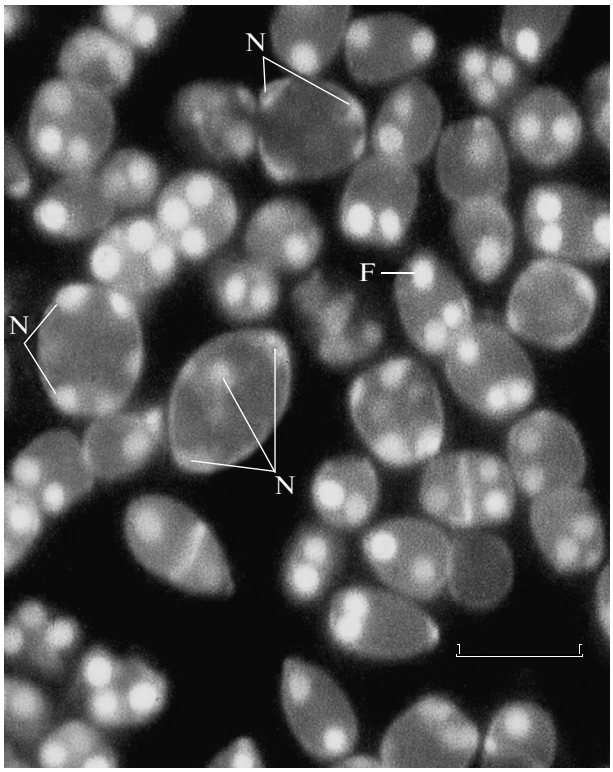

Fig. 1. Sporulating cells from a 7-day culture of *Anaerobacter polyendosporus* PS-1^T grown on agarized synthetic medium with 0.2% galactose. Epifluorescence microscopy, fluorochroming with AO. Designations: N, nucleoid; F, forespore. Bar, 5 μm.

Cryofractography. Replicas of freeze-fractured cells were obtained by the methods described by Duda et al. (2001).

The sections and replicas were examined under a JEM-100B electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

RESULTS

The first signs of the ES formation in potentially multispore cells were manifested as numerous peripherally localized nucleoids revealed by light (fluorescence and phase contrast) microscopy (Fig. 1). When stained with AO, these nucleoids fluoresce green or light-green in a luminescence microscope (Fig. 1). They are also readily stained with DAPI, as shown in our previous work (Duda et al., 2014), in which the behavior of the nuclear substance during sporogenesis was considered in more detail. In addition, one can see that green bodies with blurred edges transform into more morphologically distinct ellipsoid or spheroid bodies surrounded with an envelope and acquiring orange color. These bodies are likely to be prespores (P) or forespores (F), which is confirmed by the electron microscopy data presented below. It should be

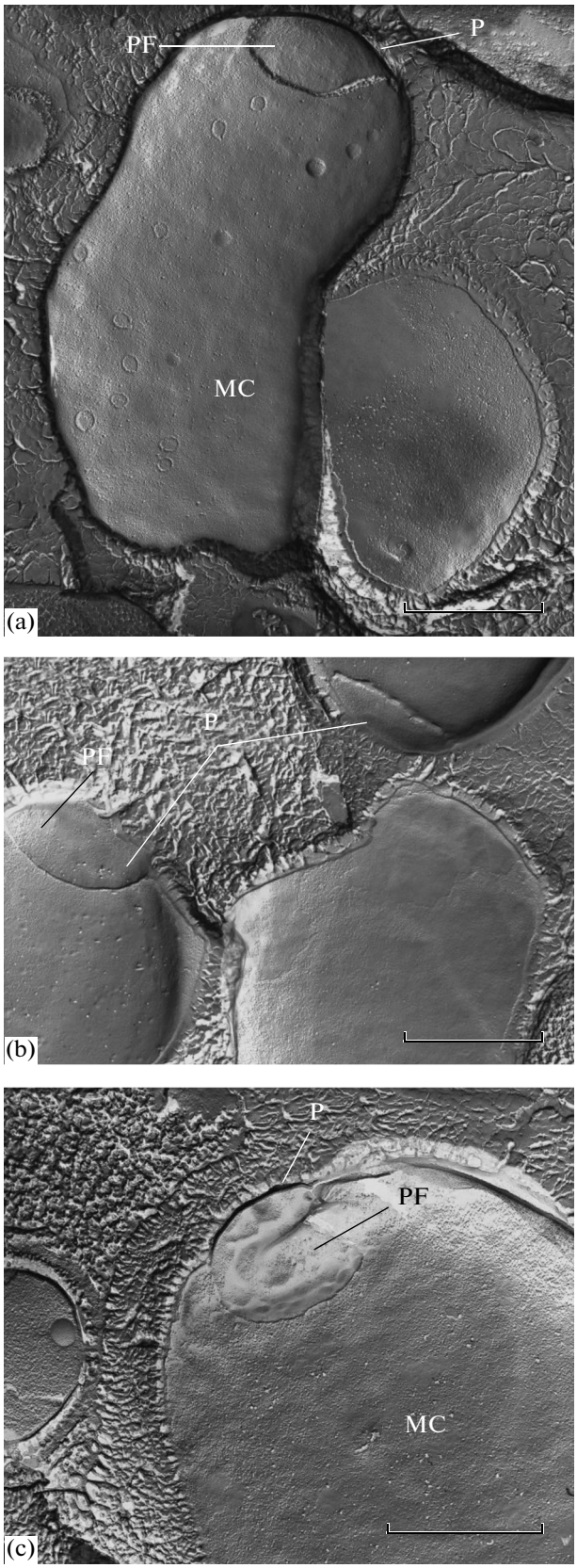

Fig. 2. PF surfaces of cryofractured membrane of prespores located at the pole of the mother cell. Electron microscopic cryofractography. Designations: P, prespore; MC, mother cell; PF, the protoplasmic face of the fracture. Bar, 1 μm.

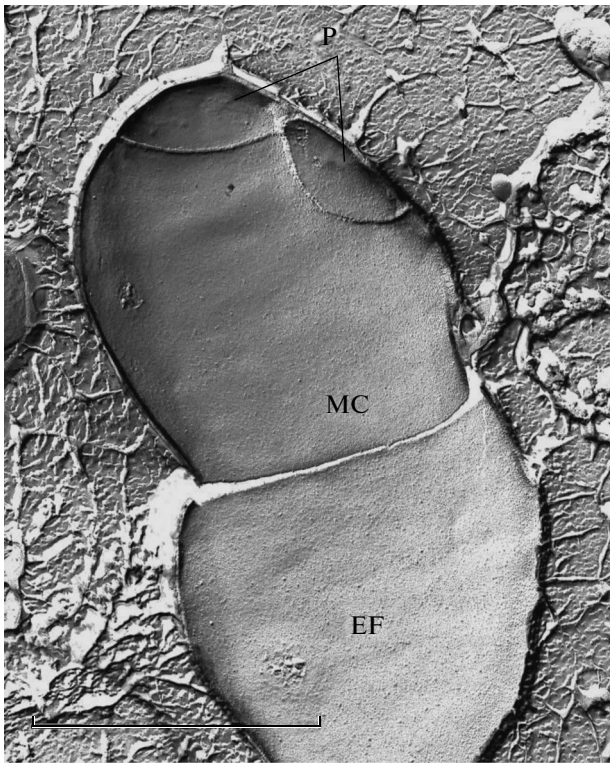

Fig. 3. Prespores (single-pole twins). Electron microscopic cryofractography. The EF surface of the membrane fracture is shown. Designations: P, prespore; MC, mother cell; EF, the exoplasmic face of the fracture. Bar, 1 μm.

noted that it is difficult to differentiate P from F under a light microscope, but they can be distinguished quite well under an electron microscope (both in cryofracture replicas and in ultrathin sections) (Figs. 2–4). In ultrathin sections, a P looks like a laterally localized nucleoid-containing cytoplasmic region separated from the cytoplasm of the large cell with a septum consisting of two three-layered membranes. The nucleoid in P appears as an electron-transparent region located in its center and filled with electron-dense DNA filaments and granules (Fig. 4). The complex of processes resulting in P formation can be considered modified cell division.

At the next stage, a forespore (F) is formed after the P has been fully surrounded by two membranes. Thus, a new cell surrounded by two elementary membranes (the inner (IM) and outer (OM)) is formed within the sporangial cytoplasm (Figs. 5–9, 11). Upon septal membrane enclosure, the formed Fs are detached from the cytoplasmic membrane of the mother cell (MC), become rounded, enlarge, and move into the MC cytoplasm (Figs. 5–9). Cell cryofractures allow visualization of the PF and EF surfaces of septal membranes, as well as the protein subunits localized in the membranes (Figs. 2, 3). Under a phase contrast microscope, forespores appear as dark bodies

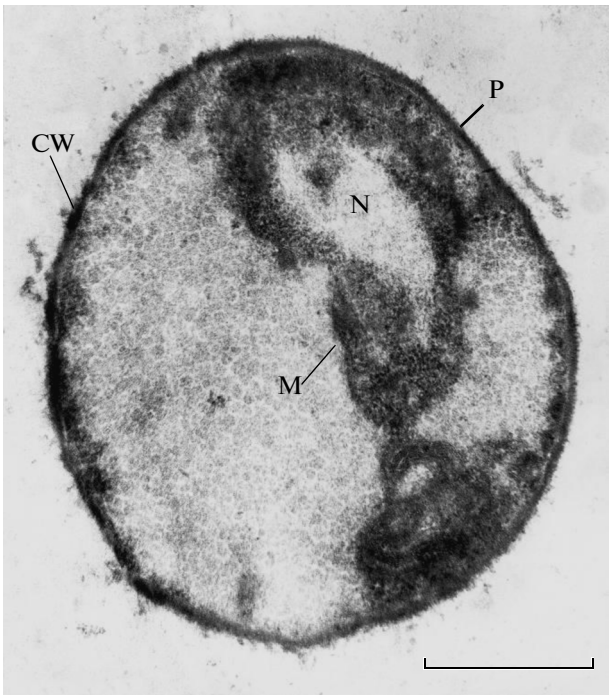

Fig. 4. Ultrathin section of a mother cell with a laterally forming prespore. Designations: P, prespore; CW, cell wall; N, nucleoid; M, the cytoplasmic membrane of the prespore. Bar, 1 μm.

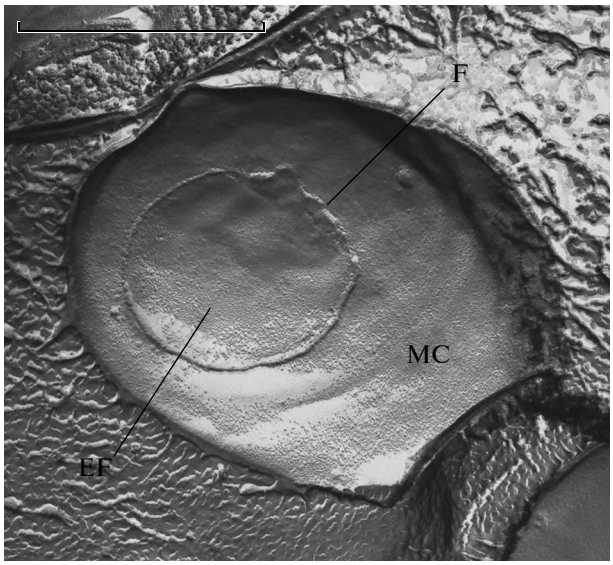

Fig. 5. Mother cell with a formed forespore. Electron microscopic cryofractography. Designations: F, forespore; EF, the exoplasmic face of the forespore fracture; MC, mother cell. Bar, 1 μm.

(Fig. 8a), and mature spores look like bright glistening structures, since, like typical spores of bacilli and clostridia, they have a high refractive index (Fig. 8b). No signs of F division inside mother cells were observed, although several thousands of F-containing cells have been examined in our experiments. Further development of the process of ES formation included the formation of spore coats (Cs), cortex (Cx), and, finally, exosporium (E) (Figs. 9–11). Their fine structure was similar to that of spore-specific structures of the bacterial endospores. The final stage was lysis of the mother cell cytoplasm; however, the sporangium cell wall (CW) of remained intact for a long time (5–10 days).

Beginning with stage 2 (P formation) and through stages 3 (F formation), 4 (cortex synthesis), 5 and 6 (formation of spore coats and exosporium), and the final stage 7 (mother cell lysis), the sequence of events during ES formation in the strain under study was analogous to that typical of the classical process of endogenous sporogenesis in bacilli and monospore clostridia (Fitz-James and Young, 1969; Long et al., 1983; Duda, 1982). It should be emphasized that the examination of several hundreds of multispore sporangia did not reveal in them forespores with any signs of division.

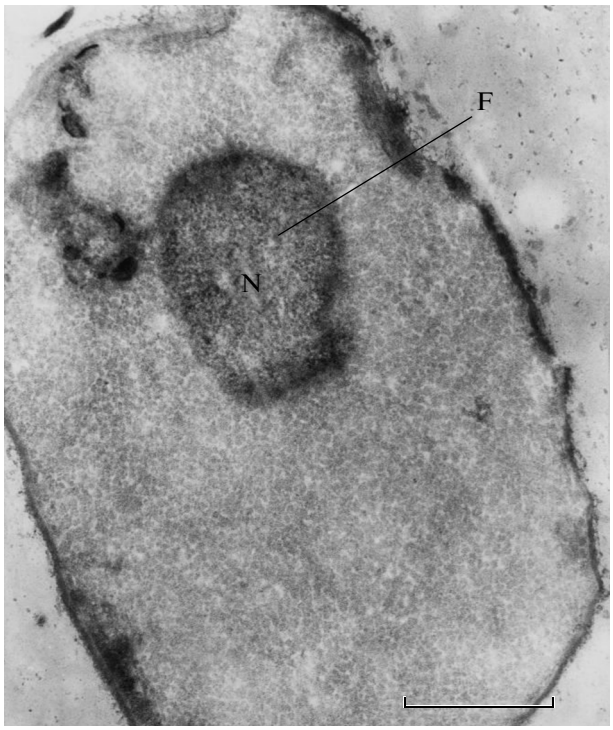

Fig. 6. Ultrathin section of a mother cell with a formed forespore. Designations: F, forespore; N, nucleoid. Bar, 1 μm.

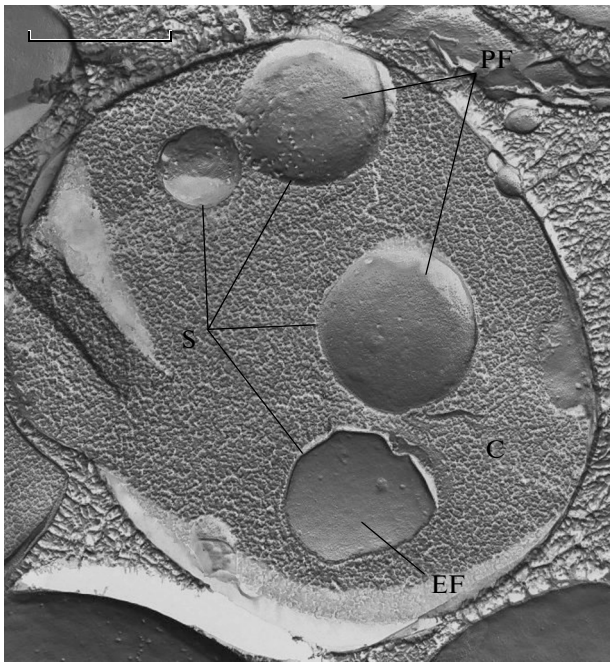

Fig. 7. Inner portion of the cytoplasm of a mother cell with four spores. Electron microscopic cryofractography. Designations: S, spore; C, cytoplasm; EF, the exoplasmic face of the spore inner membrane fracture; PF, the protoplasmic face of the spore inner membrane fracture. Bar, 1 μm.

DISCUSSION

Analysis of the data obtained in the present study leads to a conclusion that polysporogenesis in the bacterium *A. polyendosporus* PS-1^T occurs via the same cellular mechanism as that operating during endogenous sporulation in the well-studied species of bacilli and clostridia (Fitz-James and Young, 1969; Long et al., 1983; Zupancic et al., 2001; Errington, 1993). This mechanism is realized via the following sequential processes:

(a) The formation of a prespore (P) through cell division (separation of a smaller cell from a larger one);

(b) The transformation of P into a new special cell, a forespore (F), surrounded by two unit membranes (it is important to note that this process occurs via encirclement of P by the membrane of the larger (mother) cell; as a result, F gets wihin the mother cell cytoplasm; this process is termed engulfment);

(c) The synthesis and assembly of spore-specific structures: cortex, spore coats, and exosporium;

(d) The synthesis and activation of lytic enzymes, causing the degradation of the mother cell and its cytoplasm and envelope.

It should be noted that the smaller cell which is separated from the larger cell by a septum but lacks, unlike F, the outer membrane, is termed by us a prespore (P). There are substantial differences between P and F not only in the structure but also in functional properties (Duda, 1982). In case of polysporogenesis, endogenous sporulation in the bacterium under study has peculiar features. These are, primarily, the morphological changes (the cells become spherical or yeast-like), cell enlargement, and transversion of polarity of sporulating cells (Duda et al., 1987; Siunov et al., 1999; Duda et al., 2014). Based on the studies of *"Metabacterium polyspora,"* Angert and coauthors arrived at a conclusion that the emergence of numerous ES in a single sporangium of this bacterium results from multiplication of the first two forespores initially formed at the two geometrical poles of the rod-shaped mother cells (Angert and Losick, 1998; Ward and Angert, 2008). However, our thorough study of the sequence of processes of endogenous sporulation in *A. polyendosporus* PS-1^T did not reveal any signs of forespore division inside the mother cells of this bacterium. It is also essential to note that the initiation of three–five prespores in the same mother cell and further transformation of prespores into forespores occurred synchronously. As for *"Metabacterium polyspora,"* it should be noted that sporulation in this bacterium has been little studied by electron microscopy, and it cannot be excluded that polysporogenesis in *"Metabacterium polyspora"* actually occurs via a cellular mechanism analogous to that described in the present work for *Anaerobacter polyendosporus* PS-1^T.

Further progress in understanding bacterial polysporogenesis and cell biology of *Anaerobacter*

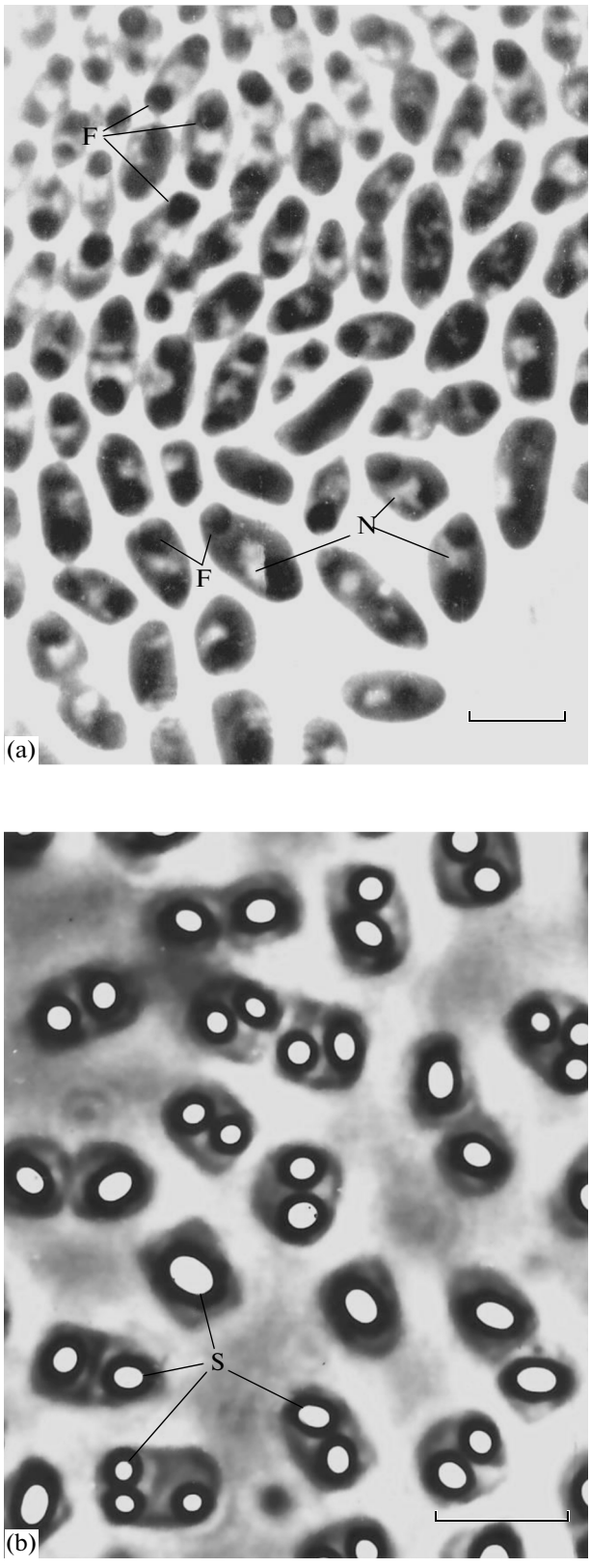

Fig. 8. Forespores (a) and mature spores (b) under a phase contrast microscope. Designations: F, forespore; S, mature spore; N, nucleoid. Bar, 10 μm.

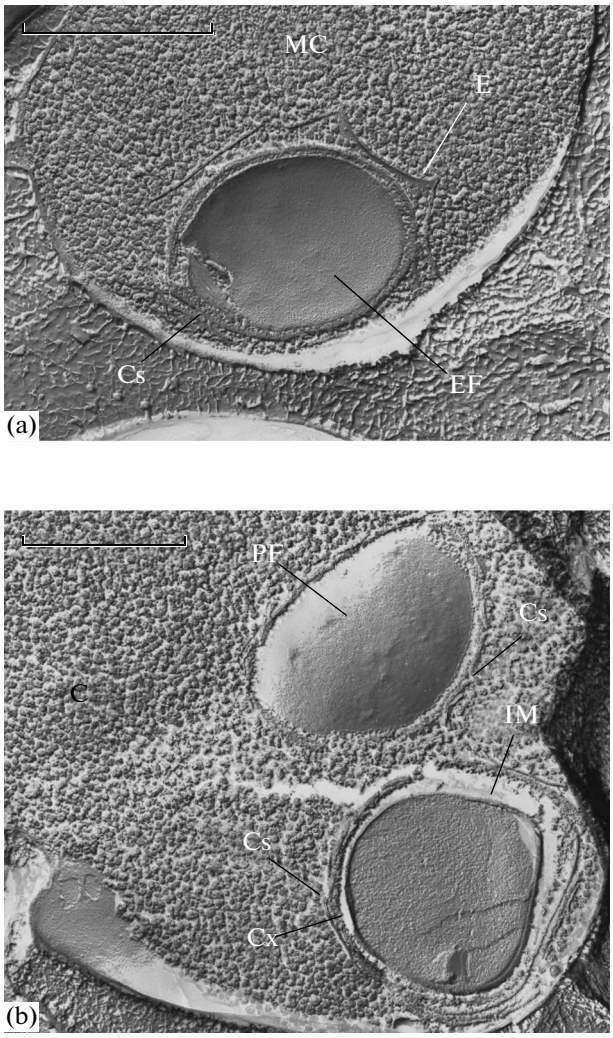

Fig. 9. Fragments of transverse cryofractures of a cell with a maturing spore (a) and a mother cell with two maturing spores that are single-pole twins (b). Electron microscopic cryofractography. Designations: EF, the exoplasmic face of a fracture of the membrane of a maturing spore; PF, the protoplasmic face of a fracture of the membrane of a maturing spore; C, the mother cell cytoplasm; Cs, spore coats; E, exosporium; Cx, cortex; IM, the spore inner membrane; MC, mother cell. Bar, 1 μm.

polyendosporus will be achieved after obtaining data on the complete nucleotide sequence of its genome, as well as after studying the genetic regulation of polysporogenesis. It is essential to understand the causes and mechanisms underlying the transversion of cell polarity and multiplication of the reproductive poles; the complex of genetic determinants of polysporogenesis should be elucidated. Answers should be given to questions that have arisen as a result of our present study, namely, the questions about the mechanism synchronizing the processes of initiation and maturation of several spores in a single spo-

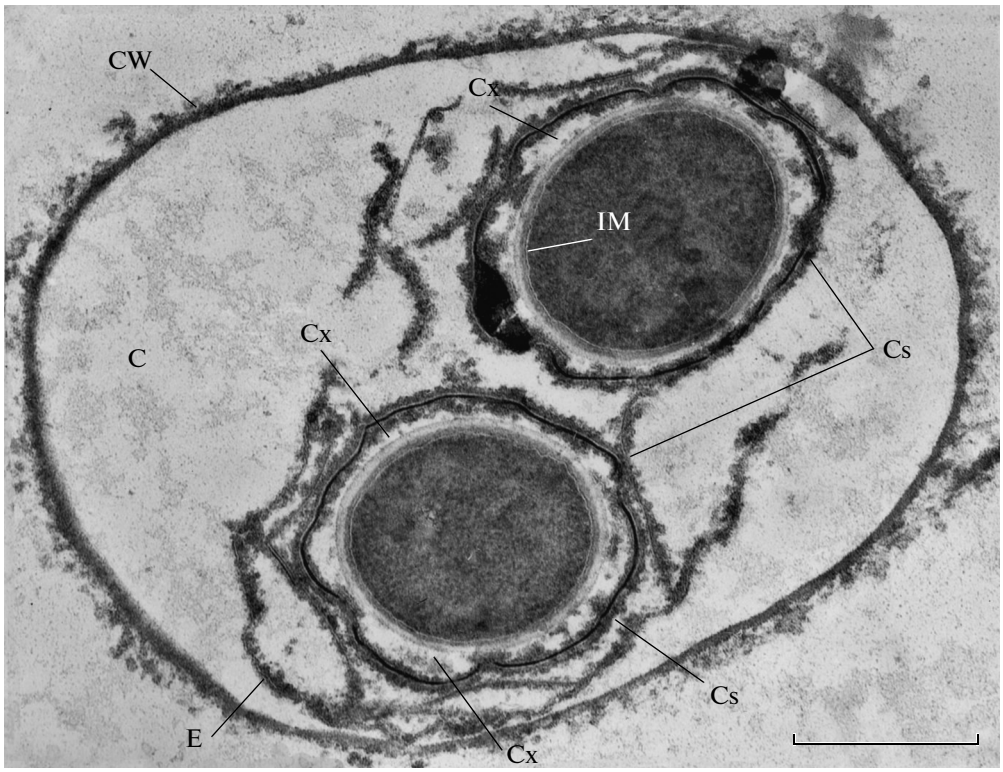

Fig. 10. Ultrathin section of a mother cell with two mature spores in the sporangium cytoplasm. Designations: CW, cell wall; C, cytoplasm; Cx, cortex; Cs, spore coats, E, exosporium; IM, the spore inner membrane. Bar, 1 μm.

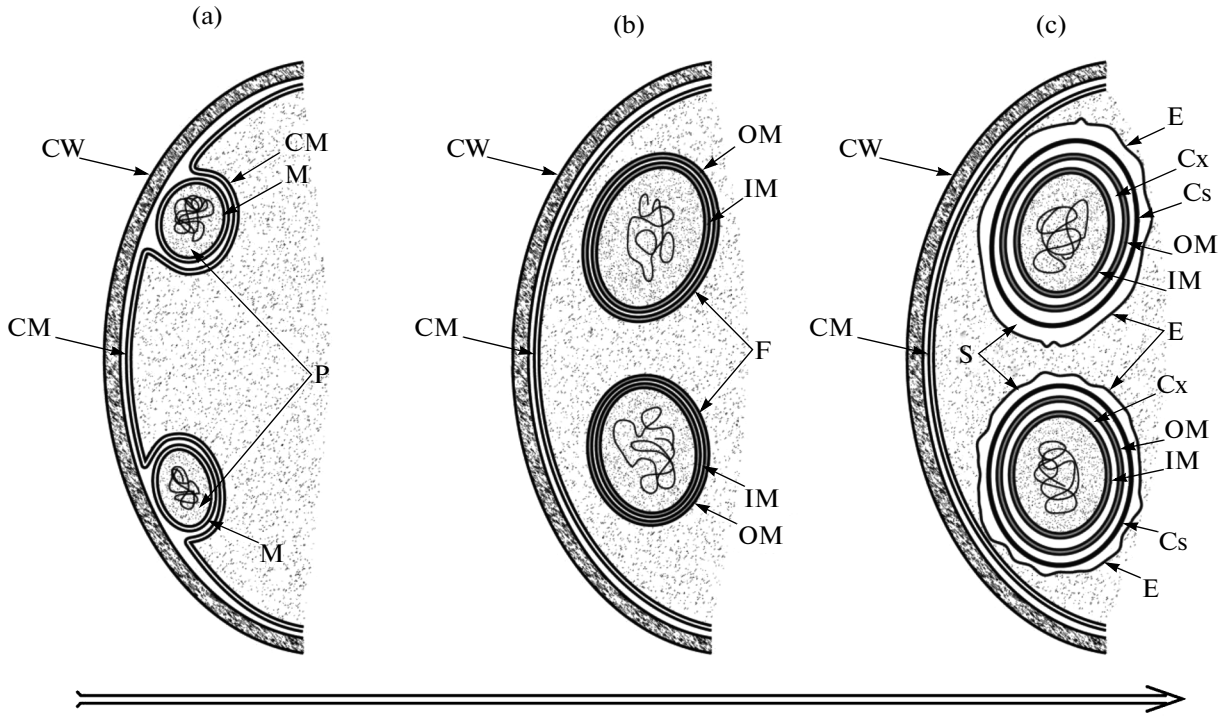

Fig. 11. Schematic representation of the sequence of processes during the formation of prespores (a), forespores (b) and spores (c) in *Anaerobacter polyendosporus* PS-1. Designations: P, prespore; F, forespore; S, spore; Cs, spore coats; M, the prespore cytoplasmic membrane; CM, the cytoplasmic membrane of the mother cell; CW, the cell wall of the mother cell; OM, the forespore or spore outer membrane; IM, the forespore or spore inner membrane; Cx, cortex; E, exosporium.

rangium and about the role of the resident genome of the mother cell in these processes.

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