ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 5, pp. 608–615. © Pleiades Publishing, Ltd., 2014.
Original Russian Text © V.I. Duda, N.E. Suzina, V.N. Polivtseva, A.B. Gafarov, A.P. Shorokhova, A.V. Machulin, 2014, published in Mikrobiologiya, 2014, Vol. 83, No. 5, pp. 575–582.

EXPERIMENTAL ARTICLES

Transversion of Cell Polarity from Bi- to Multipolarity Is the Mechanism Determining Multiple Spore Formation in *Anaerobacter polyendosporus* PS-1^T

V. I. Duda[a, b, 1], N. E. Suzina[a], V. N. Polivtseva[a], A. B. Gafarov[a], A. P. Shorokhova[a], and A. V. Machulin[a]

[a] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

[b] *Pushchino State Institute of Natural Sciences, Pushchino, Moscow oblast, 142290 Russia*

Received February 10, 2014

Abstract—The number of spores formed in a single cell of *Anaerobacter polyendosporus* PS-1^T is significantly influenced by the composition of nutrient media. Depending on carbohydrate concentration in synthetic medium, the number of spores may vary from one or two to as many as five to seven. Investigation of spore formation process by fluorescence and electron microscopy revealed that on media with 0.5–1.0% glucose or galactose most of vegetative cells remained rod-shaped after cessation of cell division in the culture. The nucleoids of these cells were localized at cell poles close to the polar site of the cytoplasmic membrane. Forespores were formed at one or both of these poles. A satellite nucleoid (operator) was observed close to each forespore. In the variant with bipolar organization of mother cells, only one or two spores per cell were formed. In the second variant of culture development, when the cells were grown at low galactose concentrations (0.1–0.3%), most of vegetative cells increased in volume and became oval or spherical after cessation of cell division in the culture. Epifluorescence microscopy with nucleic acid-specific fluorochromes (DAPI and acridine orange) revealed the presence of multiple (six to nine) nucleoids in these cells. The nucleoids were located at the cell periphery in close contact with the cytoplasmic membrane. These nucleoids became the centers (poles) for forespore formation. Thus, in the early stationary phase transversion from bipolar to multipolar cells occurred. Cessation of cell division combined with continuing replication of the nucleoids resulted in formation on multinuclear cells. The multiplicity of nucleoides and multipolarity of these cells were prerequisites determining endogenous polysporogenesis, occurring as synchronous formation of three to seven twin spores in many of the oval and spherical cells.

Keywords: cell polarity, polarity transversion, ultrastructural organization of bacteria, nucleoid, polynuclearity (multiple nucleoids), endogenous sporogenesis, polysporogenesis, twin spores, polymorphism

DOI: 10.1134/S0026261714050105

Most bacteria usually form only one spore per cell in the course of endogenous sporulation. The formation of several (up to eight) endospores in a single cell has been described for two genera of anaerobic bacteria only: *"Metabacterium"* [1, 2] and *Anaerobacter* [3]. Our works have demonstrated that bacteria of the genus *Anaerobacter* are phylogenetically and physiologically close to representatives of the genus *Clostridium* [3, 4]. Recent studies of microorganisms of the genus *"Metabacterium"* (never isolated in a pure culture) revealed their prokaryotic nature and affiliation with the phylogenetic group of clostridia [5]. The soil bacterium *Anaerobacter* is a suitable object for studying polysporogenesis because one of the members of this genus, *A. polyendosporus* strain PS-1^T, has been isolated and studied in pure culture [3, 4, 6, 7]. The nature of bacterial polysporogenesis has not been adequately studied so far, either in terms of cytological mechanisms or in respect to genetic regulation of this process.

The present work is devoted to electron-microscopic and luminescence-microscopic studies of the sporulation process in *A. polyendosporus* PS-1^T on media with different carbohydrate content.

MATERIALS AND METHODS

Research objects. In this study, we used strain *A. polyendosporus* PS-1^T, described by us in detail earlier [3]. The bacterium was cultivated on potato agar (PA) and liquid (LSM) and agarized (ASM) synthetic media proposed previously by Pfennig [8]. LSM and ASM were supplemented with trace elements according to Pfennig and Lippert [9]; 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 liquid

[1] Corresponding author; e-mail: duda@ibpm.pushchino.ru

medium were grown using the Hungate technique [10], while those on agarized medium were incubated in anaerobic jars filled with oxygen-free N_2 (95%) and CO_2 (5%).

Phase contrast microscopy. Phase contrast microscopy used OPTON ICM 405 (Zeiss, Germany) and LUMAM (LOMO, Russia) microscopes.

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

The percentage of polysporous cells was determined by counting the total number of sporulating cells in a culture and the numbers of two-, three-, four-, five-, six- and seven-spore cells and then calculating the percentage of each fraction. A total of 150 cells was considered. The cells were counted directly in the microscope field or on micrographs of microscope fields; the total cell number in the cultures was not counted.

The percentage of cells with six to seven spores was determined in hermetical microscopic chambers that provide the opportunity to use the methods employed for cell count in Thoma and Goryaev chambers; in doing this, the whole area of the chamber was scanned.

Electron-microscopic methods. Ultrathin sections were prepared as follows: the cells were concentrated by centrifugation (10000 *g*, 15 min) and fixed in a 2.5% glutaraldehyde solution in 0.05 M cacodylate buffer (pH 7.2) for 1 h at 4°C. Then, the material was 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 embedded in agar, dehydrated in a series of alcohols of increasing concentration, and embedded in Epon 812. The ultrathin sections, obtained with an LKB 2128 ultramicrotome (LKB Produkter, Sweden), were stained with 3% uranyl acetate solution in 70% ethanol and additionally contrasted with lead citrate according to Reynolds [12]. The specimens were examined under a JEM 100B electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

Genome size determination. Genome size was determined based on the methodical principles described in [13], using restriction endonucleases and gel electrophoresis. The large amount of polysaccharide formed in the PS-1^T cells grown on all types of the nutrient media used in this work substantially impeded the isolation of DNA preparations suitable for analysis. Therefore, DNA was isolated from cell suspensions that had been stored under anaerobic conditions in a carbohydrate-free LSM medium for 1–2 days at 28°C.

Native chromosomal DNA was treated with restriction endonucleases produced by Fermentas (Lithuania) according to the manufacturer's instructions. For accurate measurement of the PS-1^T genome size, restriction analysis employed eight enzymes recognizing AT- or GC-rich sequences of 6 or 8 pairs of nucleotides.

As a result the analysis performed, three enzymes (*Apa*I, *Sgs*I, and *Not*I) yielding the lowest number of restriction fragments were selected for further genomic analysis by inversion and pulse-field electrophoreses.

Native chromosomal DNA immobilized in agarose was obtained according to the FIGE Mapper Instruction Manual and Application Guide (BioRad, United States).

The sizes of DNA fragments of 5 to 150 kb were determined by inversion electrophoresis in a FIGE Mapper (BioRad, United States). Electrophoresis was carried out according to the manufacturer's instructions by program no. 8 at room temperature in 0.5× Tris-borate buffer for 20 h at a direct voltage of 180 V and a reverse voltage of 120 V, with 0.4–3.5 s pulses.

The sizes of DNA fragments larger than 200 kb were determined by pulse-field electrophoresis (PFGE) in a Pulsaphor System (Pharmacia LKB, Sweden). Electrophoresis was carried out in 1% agarose by program no. 2: at 14°C in 0.5× Tris-borate buffer (2.5 L) for 24 h at 180 V, with 5–30 s pulses.

RESULTS

The *A. polyendosporus* PS-1^T cultures grown on PA, LSM and ASM were characterized by two cell morphotypes: rod-shaped (cylindrical) cells (RCs) and large oval (spherical) cells (OCs). RCs prevailed throughout the whole cycle of development on the above media supplemented with 0.5–1.0% glucose or galactose (Figs. 1, 3, and 7, *1–3*); usually they formed chains of two to four cells. OCs were predominant on LSM and ASM supplemented with low concentrations of carbohydrates: 0.1– 0.3% glucose or galactose (Fig. 2a). One or two endospores (ES) were formed in RCs upon transition to the stationary phase of culture development on LSM and ASM with high concentrations of carbohydrates (0.5–1.0% glucose or galactose) (Figs. 1b and 7, *2, 3*). ES formation began at the poles of mother cells close to the polar site of the cytoplasmic membrane. Nucleoids were also located at

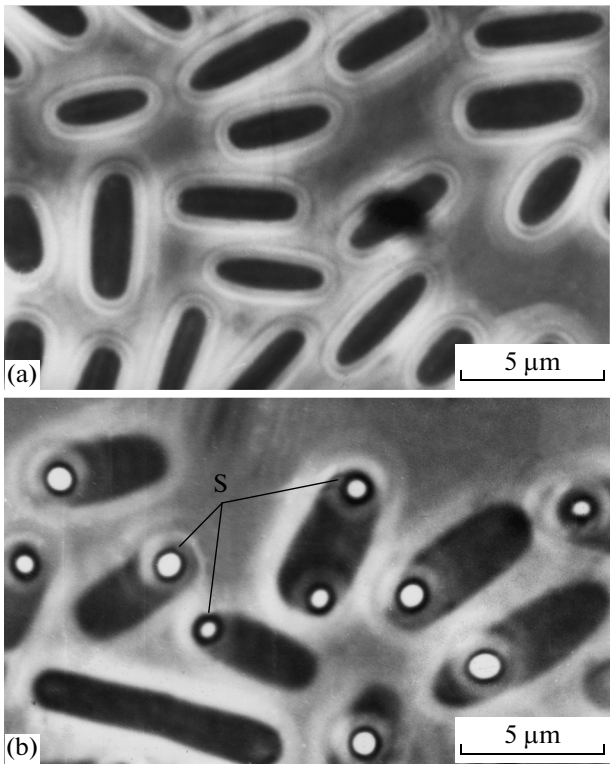

Fig. 1. Vegetative cells from a 3-day culture grown in LSM with 0.5% galactose (a) and sporulating cells from a 5-day culture grown in the same medium (b). Phase contrast microscopy. S, spore. Bar, 5 μm.

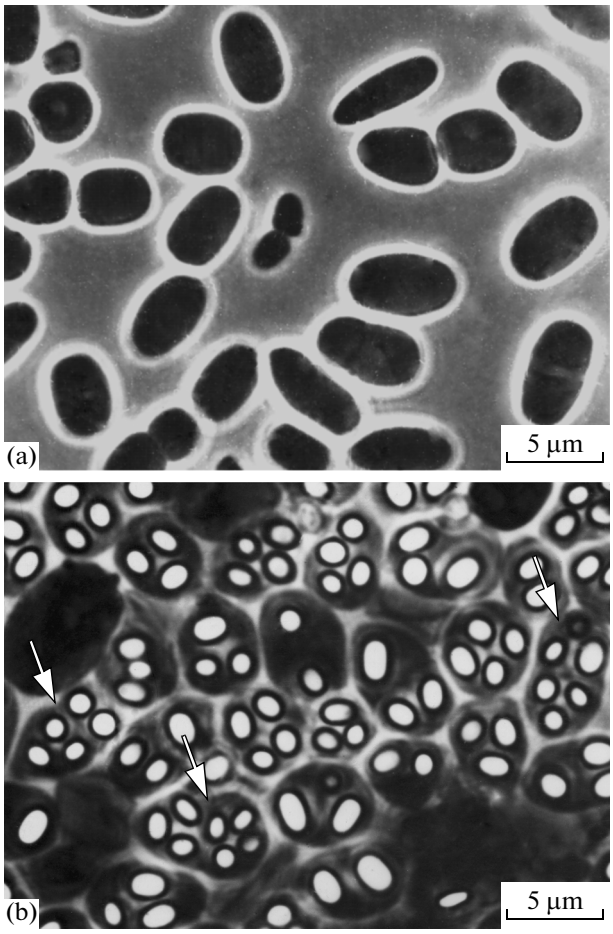

Fig. 2. Vegetative cells from a 5-day culture grown in LSM with 0.1% galactose at the stage of cessation of cell division and transition to sporulation (a) and sporulating cells from a 15-day culture grown in the same medium (b). The arrows indicate sporangia with five to seven spores. Phase contrast microscopy. Bar, 5 μm.

these sites. The percent ratio of two- and three-spore cells to the total cell number under these conditions was 25–40% and <0.1%, respectively. At the same time, on LSM and ASM with low concentrations of carbohydrates (0.1 to 0.3% glucose or galactose), many OCs formed three to seven ESs (Figs. 2b and 7, *8–11*). The data presented in the table show that two-spore sporangia were predominant (52%) in the culture grown in LMS with 0.2% galactose, but the quantity of mother cells with three ESs was also considerable (12%). The sporangia with four or five ESs made up ~1.5%, while those with six or seven ESs accounted for less than 0.01%. It should be noted that sporangia with six to seven spores were formed in LSM only and could be found only in the films formed by the culture at the bottom of the tubes. Galactose had an activating effect on spore formation: in media with this carbohydrate, the percentage of sporangia with two to five spores was higher by ~20% compared to media with low concentrations of glucose. No multispore cells could be revealed by microscopy of cultures grown on other media.

On the other hand, it should be noted that high concentrations (1.5–2%) of the tested carbohydrates in PA, LSM, and ASM completely inhibited spore formation in strain PS-1^T. At the same time, the following processes were observed on PA supplemented with 0.1–0.2% glucose or galactose: (a) transformation of many rod-shaped cells into thickened rounded cells

Percentage of mother cells with different numbers of spores in samples of a culture grown in LSM with 0.2% galactose for 15 days

Sporangia with different numbers of spores, %				
one spore	two spores	three spores	four or five spores	six or seven spores
34.5	52	12	1.5	<0.01

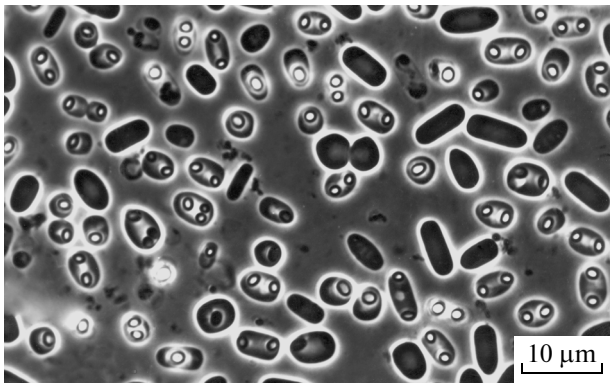

Fig. 3. Sporulating cells from a 10-day culture grown on PA with 0.1% galactose. It can be seen that many sporangia contain two spores. Phase contrast microscopy. Bar, 10 μm.

and (b) sporulation with the formation of ~1–1.5% five-spore cells (Fig. 3). However, sporangia with six or seven ESs were not found on this medium.

Epifluorescence microscopy with DNA-specific staining by DAPI or AO showed the presence of two nucleoids in RCs in the beginning of sporulation, and their division resulted in the formation of ESs and adjacent satellite nucleoids (Figs. 4 and 7, *1–3*). In spherical cells, after their enlargement and the cessation of division (septa were absent), the nucleoids continued to divide, being located along the periphery of cells in close contact with the cytoplasmic membrane. One spherical cell could contain 4 to 11 discrete nucleoids (Figs. 5 and 7, *4–7*). The diameters of nucleoids in spherical cells were ~0.3–0.6 μm (in rod-shaped cells, they were 2- to 3-fold larger). The small sizes of nucleoids correlate with the small genome size of the strain PS-1^T: ~2.4 Mb (no plasmid DNA was revealed under the experimental conditions used). Then, the process of sporulation occurred in the spherical cells. Formation of forespores began in the sites of localization of peripheral nucleoids. After the forespores were formed, they synchronously turned into mature refractile ESs, the maximum number of which could reach seven (Fig. 2b). Electron microscopy of ultrathin sections demonstrated that the engulfment (formation of the forespore membrane and the capture of nucleoid by it) was a lateral process in the oval/spherical cells (Figs. 6a, 6b). It should be noted that all of the twin ESs in a particular multispore sporangium were formed synchronously. The synchronicity of these processes is evidenced by the occurrence of twin spores at the same stage of sporulation and the simultaneous successive appearance in them of such spore-specific structures as spore coats, cortex, and exosporium (Fig. 6c). The ESs formed in multispore sporangia were in most cases lesser in size than the ESs formed in mono- and two-spore mother cells; however, microscopic analysis has shown that they germinate well when transferred onto PA.

The third *A. polyendosporus* PS-1^T cell morphotype (polygonal cells, PGC) has been described by us previously [4, 7]. PGC are sporadically formed in old (15- to 20-day) cultures grown on PA supplemented with 1–2% glucose or galactose. This morphotype may reach 20–30% of the total cell number in the cultures. However, this phenomenon is not observed regularly, and further studies are needed to find out the conditions and factors that induce PGC formation.

It should be noted that the detection of nucleoids in sporulating cells, as well as DNA isolation from them, was severely hindered by the occurrence in their cytoplasm of a diffusely distributed polysaccharide, apparently granulose, as has been shown for some species of clostridia [14]. The polysaccharide imparts osmiophobicity and electron transparency to the cytoplasm and masks the nucleoplasm (Fig. 6b). This polysaccharide is stained brown or dark-blue with iodine. At the late stages of sporulation, as the spores maturate, the cytoplasm on ultrathin sections resumes its normal appearance typical of the cytoplasm of vegetative cells.

DISCUSSION

The results of our study demonstrate high sensitivity of strain *A. polyendosporus* PS-1^T to the catabolite repression of sporulation by excessive amounts of carbohydrates in the medium. High carbohydrate concentrations (1.5–2%) in the medium inhibit sporulation, while low concentrations (0.1–0.3%) activate this process, resulting not only in abundant sporulation but also in polysporogenesis, i.e., formation of several (three to seven) ESs in one cell. It is still unclear why polysporogenesis does not occur in all of the cells. Cytological analysis by epifluorescence and electron microscopy leads to a conclusion that polysporogenesis is based on the phenomenon of transition of cells from bipolarity to multipolarity, a process which we have termed *transversion* (i.e., choice by cells of a different version of polarity). Development of

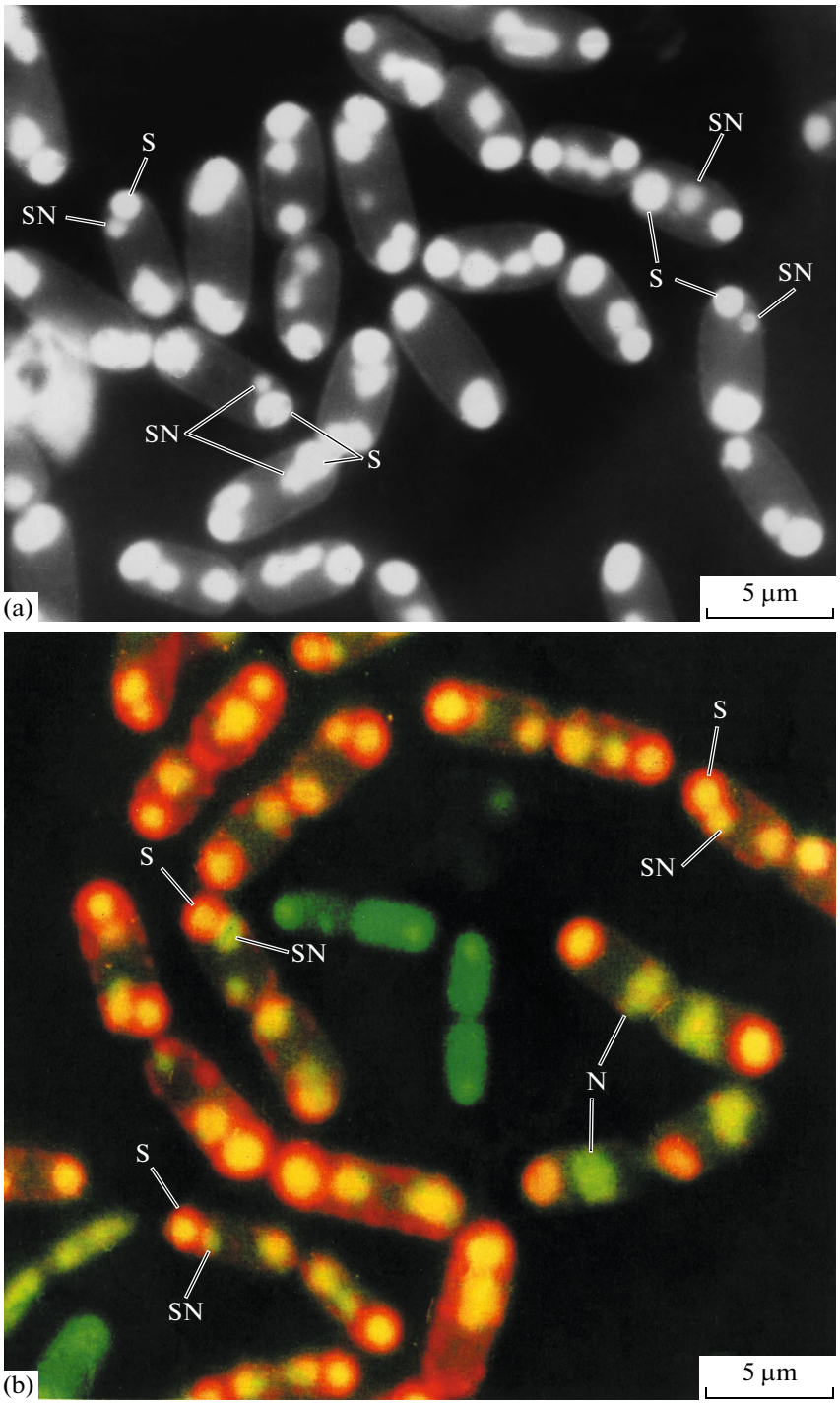

Fig. 4. Sporulating cells from a 8-day culture grown in LSM with 0.5% galactose (a, b). Epifluorescence microscopy. The specimen was fluorochromated with AO. S, spore; N, nucleoid; SN, satellite nucleoid. Bar, 5 μm.

multipolarity is determined by the continuing division of nucleoids after cessation of the division of cells and their transformation into large oval and spherical cells. In these cells, numerous nucleoids are localized along the cell periphery, in close contact with the cytoplasmic membrane. They become the new poles of the cells and the centers of ES formation. It has been shown that DNA synthesis in clostridia may continue during sporulation [15]. This seems to be true also for the bacterium *A. polyendosporus* PS-1^T, which belongs

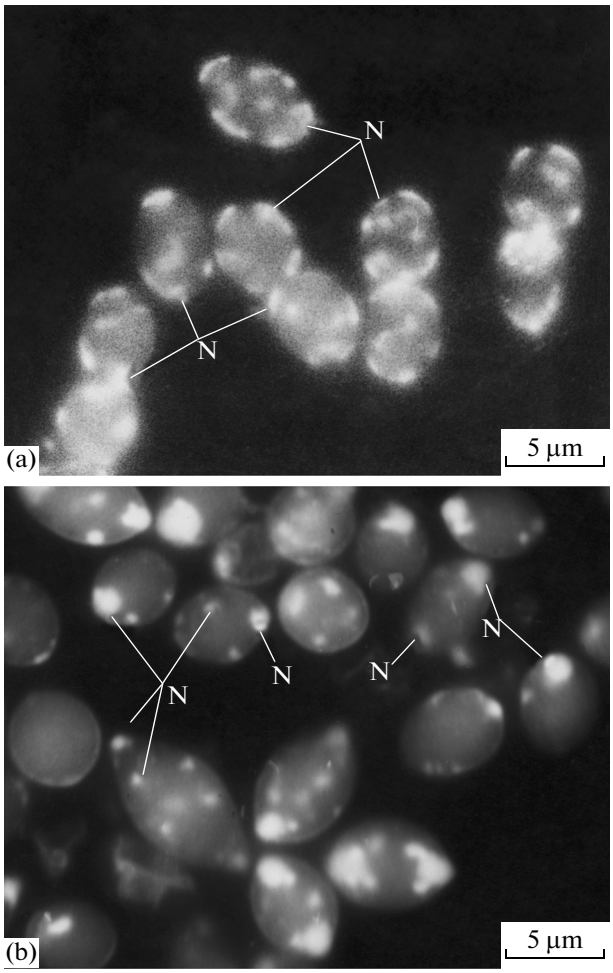

Fig. 5. Spherical and oval cells before sporulation in a 10-day culture grown in LSM with 0.1% galactose. Epifluorescence microscopy. The specimens were fluorochromated with DAPI (a) or AO (b). N, nucleoid. Bar, 5 μm.

to clostridia [4]. Cell polarity is one of the basic principles of cell organization in both eukaryotes and prokaryotes [16–20]. The poles play an important role in cell morphogenesis and sporulation, acting as geometrical regulators of these and some other processes. The poles, cellular sites of peculiar structure and function, are formed de novo during cell division. Several works showed the poles to be the cell regions of concentration of the proteins necessary for chromosomal DNA attachment to the cytoplasmic membrane, of enzymes controlling DNA replication, and of cytoskeletal and signaling proteins [16–20]. These regions are most often pronounced morphologically; however, the present study shows that it not always the case. In the spherical cells of *A. polyendosporus* PS-1^T, multiple poles are cytologically distinct only due to the presence of nucleoids in them. Near the spores, in close contact with their envelopes, satellite nucleoids are localized. It may be supposed that the function of these nucleoids is to regulate the processes occurring in the vicinity of the spores, i.e., in the cytoplasm surrounding them. It is still unclear whether the multispore cell has a resident, superior nucleoid that coordinates the activities of all other nucleoids. The existence of such coordinator nucleoid is supposed by synchronicity of the formation stages of many spores in a single sporangium. However, the coordinator nucleoid was not detected cytologically, and it seems necessary to develop fundamentally novel approaches and methods to prove this assumption.

The study of the *A. polyendosporus* PS-1^T genome size has shown that, in spite of the large size of the cells, the genome of this bacterium is small, ~2.4 Mb. Most probably, dimensions of the cells are associated not only with their genome size but also with their ploidy. It has been shown [21] that the giant anaerobic bacterium *Epulopiscium* is characterized by extreme polyploidy: its cells contain tens of thousands of genome copies.

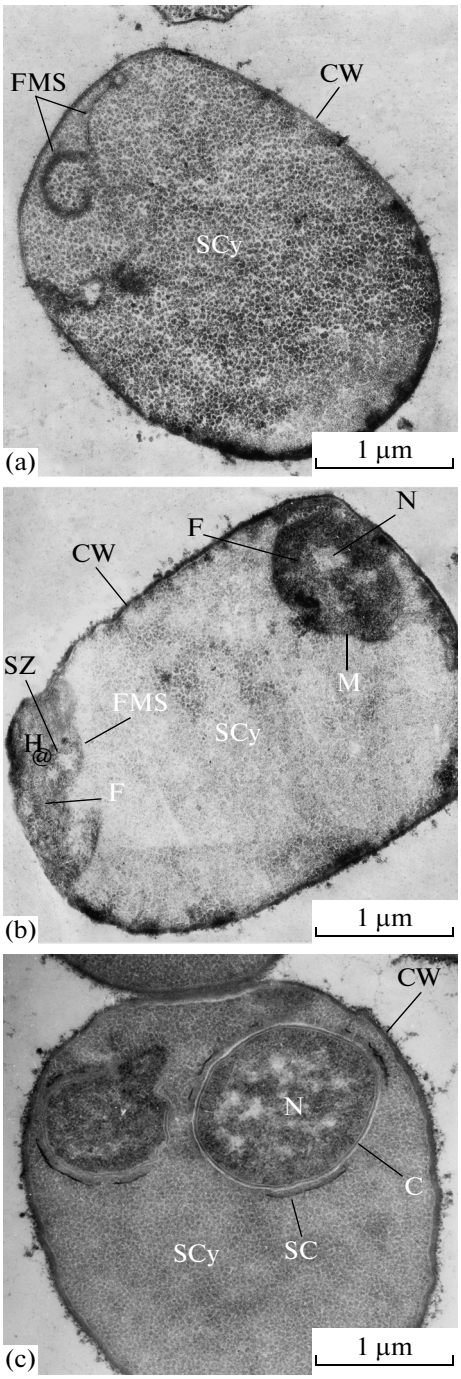

Fig. 6. Ultrathin sections of sporulating cells from cultures grown in LSM with 0.1% galactose for 12 (a, b) and 15 (c) days. Figure 6a shows a forespore membrane septum in the process of formation; Fig. 6b shows two forespores (at the same maturation stage) in the opposite regions of sporangium. FMS, forespore membrane septum; CW, cell wall; SCy, sporangium cytoplasm; F, forespore; SC, spore coats; N, nucleoid; SZ, sporogenic zone; M, forespore membrane; C, cortex. Bar, 1 μm.

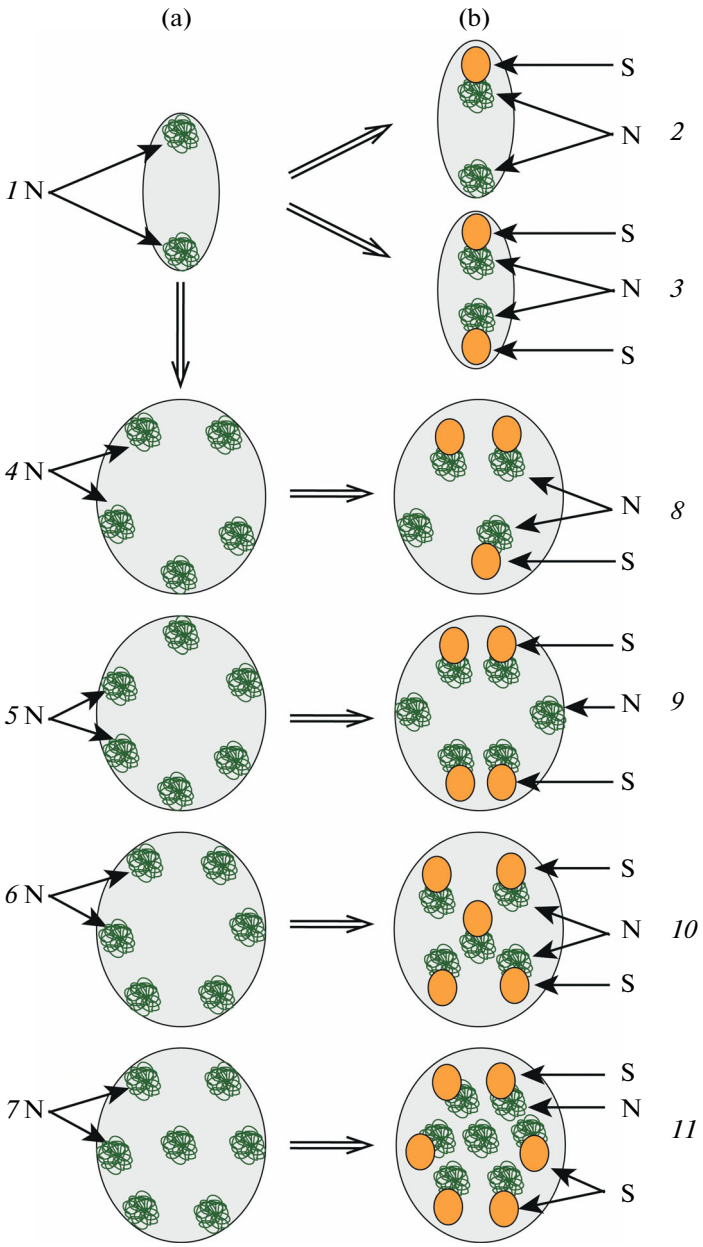

Fig. 7. Schematic representation of sporulating cells and localization of nucleoids and endospores in these cells during development in LSM with 0.5–1.0% galactose (*1–3*) and in LSM with 0.1–0.3% galactose (*4–11*): (a) a series of cells at the first stage of sporulation; (b) a series of cells with spores formed. The double arrows indicate the vectors of process development. S, spore; N, nucleoid.

ACKNOWLEDGMENTS

We are grateful to the senior engineer Lylia P. Chigaleichik for assistance in growing the microbial cultures.

This work was performed within the framework of the State Research Assignment for 2013 from the

Ministry of Education and Science of the Russian Federation.

REFERENCES

1. Chatton, E. and Pérard, C., Schizophytes du caecum de cobaye, *"Metabacterium polyspora"* n.g., n.sp, *Compendens Rendus Hebdomadaires Societe de Biology* (Paris), 1913, vol. 74, pp. 1232–1234.
2. Krasil'nikov, N.A., Microbes from the guinea-pig intestine: *Oscillospira guilliermondii* and *Metabacterium polyspora, Mikrobiol. Zh.*, 1928, vol. 6, p. 247.
3. Duda, V.I., Lebedinsky, A.V., Mushegjan, M.S., and Mitjushina, L.L., A new anaerobic bacterium, forming up to five endospores per cell—*Anaerobacter polyendosporus* gen. et spec. nov, *Arch. Microbiol.*, 1987, vol. 148, pp. 121–127.
4. Siunov, A., Nikitin, D., Suzina, N., Dmitriev, V., Kuzmin, N., and Duda, V., Phylogenetic status of *Anaerobacter polyendosporus*, an anaerobic, polysporogenic bacterium, *Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 1119–1124.
5. Angert, E.R., Brooks, A.E., and Pace, N.R., Phylogenetic analysis of *Metabacterium polyspora*: clues to the evolutionary origin of daughter cell production in *Epulopiscium* species, the largest bacteria, *J. Bacteriol.*, 1996, vol. 178, pp. 1451–1456.
6. Duda, V.I., Suzina, N.E., Severina, L.O., Dmitriev, V.V., and Karavaiko, G.I., Formation of flat lamellar intramembrane lipid structures in microorganisms, *J. Membr. Biol.*, 2001, vol. 180, pp. 33–48.
7. Duda, V.I., Suzina, N.E., and Dmitriev, V.V., Ultrastructural organization of the cytoplasmic membrane of *Anaerobacter polyendosporus* as evidenced by electron microscopic cryofractography, *Microbiology* (Moscow), 2001, vol. 70, no. 6. pp. 657–666.
8. Pfennig, N., Anreicherungskulturen für rote und grüne Schwefelbakterien, *Zbl. Bakt. I. Abt. Orig. Suppl. 1,* 1965, pp. 179–189.
9. Pfennig, N. and Lippert, K.D., Uber das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien, *Arch. Microbiol.*, 1966, vol. 55, pp. 245–256.
10. Hungate, R.E., *The Rumen and Its Microbes*, New York–London: Academic Press, 1966.
11. Reynolds, E., The use of lead citrate at high pH as an electron opaque stain in electron microscopy, *J. Cell Biol.*, 1963, vol. 17, pp. 208–212.
12. Janel, S.R., Hector, J.S.R., and Johnson, A.R., Determination of genome size of *Pseudomonas aeruginosa* by PFGE: analysis of restriction fragments, *Nucleic Acids Res.,* 1990, vol. 18, pp. 3171–3174.
13. Duda, V.I., Cytological peculiarities of spore-forming bacteria, *Usp. Mikrobiol.*, 1982, no. 17, pp. 87–116.
14. Labbe, R.G. and Duncan, C.L. Synthesis of deoxyribonucleic acid, ribonucleic acid, and protein during sporulation of *Clostridium perfringens, J. Bacteriol.,* 1976, vol. 125, no. 2, pp. 444–452.
15. Goehring, N.V. and Grill, S.W., Cell polarity: mechanochemical patterning, *Trends Cell Biol.*, 2013, vol. 23, no. 2, pp. 72–80.
16. Shapiro, L., McAdams, H.H., and Losick, R., Generating and exploiting polarity in bacteria, *Science*, 2002, vol. 298, pp. 1942–1945.
17. Hamamoto, T., Takashina, T., Grant, D.W., and Horikoshi, K., Asymmetric cell division of a triangular halophilic archaebacterium, *FEMS Microbiol. Lett.*, 1988, vol. 56, pp. 221–224.
18. Ben-Yehuda, S., Rudner, D.Z., and Losick, R., Rac, A., a bacterial protein that anchors chromosomes to the cell poles, *Science*, 2003, vol. 299, pp. 532–536.
19. Jacobs, C., Domian, I.J., Maddock, J.R., and Shapiro, L., Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division, *Cell*, 1999, vol. 97, no. 1, pp. 111–120.
20. Mendell, J.E., Clements, K.D., Choat, J.H., and Angert, E.R., Extreme polyploidy in a large bacterium, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, vol. 105, p. 6730–6734.

Translated by E. Makeeva