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**EXPERIMENTAL ARTICLES**

# Ultrastructural Organization of the Cytoplasmic Membrane of *Anaerobacter polyendosporus* as Evidenced by Electron Microscopic Cryofractography

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**Abstract**—*Anaerobacter polyendosporus* cells do not have typical mesosomes. However, the analysis of this anaerobic multispore bacterium by electron microscopic cryofractography showed that its cytoplasmic membrane contains specific intramembrane structures in the form of flat lamellar inverted lipid membranes tenths of nanometers to several microns in size. It was found that these structures are located in the hydrophobic interior between the outer and inner leaflets of the cytoplasmic membrane and do not contain intramembrane particles that are commonly present on freeze-fracture replicas. The flat inverted lipid membranes were revealed in bacterial cells cultivated under normal growth conditions, indicating the existence of a complex-type compartmentalization in biological membranes, which manifests itself in the formation of intramembrane compartments having the appearance of vesicles and inverted lipid membranes.

*Key words*: microbial membranes, intramembrane structures, inverted lipid membranes, microbial cell ultrastructure.

The present-day concept of the structure of biomembranes is based on the idea of a lipid bilayer as the main structural unit of eukaryotic and prokaryotic membranes, although it is known that the hydrophobic portion of the cytoplasmic membranes of some archaea is made up of a single layer of specific bipolar lipids [1]. Furthermore, there is evidence that lipid molecules may form nonbilayer structures in some regions of normal bilayer biomembranes in the form of hexagonal HI and HII phases, cubic and rhombic particles, and inverted lipid micelles and vesicles [2–4]. It should, however, be noted that most of these observations were made in experiments with artificial membranes, whereas little is known about the formation of nonbilayer lipid structures in natural biomembranes.

When studying the ultrastructure of the multispore anaerobic bacterium *Anaerobacter polyendosporus* [5], we observed the formation of inverted lamellar lipid structures in some regions of its cytoplasmic membrane (CM). Similar intramembrane structures were also observed in the cytoplasmic membrane of *Sulfobacillus thermosulfidooxidans* [6].

The present work is a continuation of the study of the ultrastructural organization of the cytoplasmic membrane of *An. polyendosporus* by the electron microscopic cryofractography technique.

## MATERIALS AND METHODS

**Bacteria and cultivation conditions.** Two bacterial strains were used in this study. (1) *Anaerobacter polyendosporus* strain PS-1 is an obligately anaerobic, mesophilic, heterotrophic bacterium capable of forming up to 6–7 endospores per cell. In physiology and phylogeny, this bacterium is close to saccharolytic species of the genus *Clostridium* [5, 7]. (2) *Clostridium* sp. strain 15, which was isolated from soil, is an anaerobic pectinolytic bacterium close to *Clostridium acetonobutylicum* in many morphological, physiological, and biochemical properties. Both bacteria were grown anaerobically at 28°C on potato agar or in a chemically defined medium with glucose as the sole source of carbon and energy [7].

**Preparation of freeze-fracture replicas.** Freeze-fracturing was carried out in a JEE-4X vacuum evaporator equipped with a device that provided the rapid quenching of microbial cells at a rate of about $10^6$ °C/s [8]. The preliminary steps of cryofixation (the centrifugation of cells and their transfer into the evaporator) were carried out either at room temperature (20 °C) or at the cultivation temperature (28 °C). The biomass was frozen in liquid propane overcooled to –196°C with liquid nitrogen and freeze-fractured when the temperature of the sample reached –100°C at a pressure of $3 \times 10^{-4}$ Pa. The replicas of freeze-fractured cells were prepared by means of the vacuum deposition of a platinum–carbon

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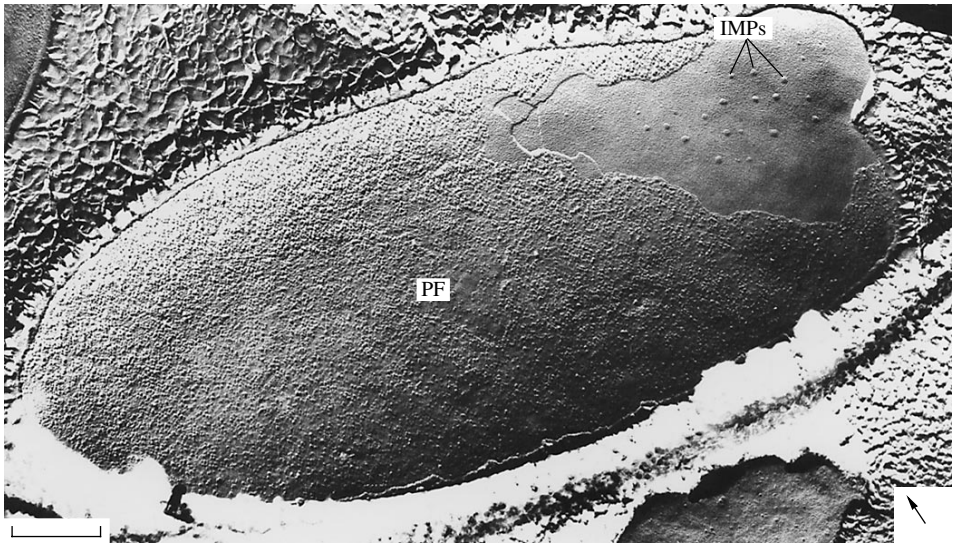


**Fig. 1.** Freeze-fracture replica (PF face) of the cytoplasmic membrane (CM) of *An. polyendosporus* strain PS-1 grown on potato agar. The smooth polar region of the CM accommodates sparse large intramembrane particles (IMPs). The arrowhead in this and other figures shows the shadow direction. Bar = 0.5 μm.

mixture at an angle of 30° followed by carbon deposition at an angle of 90°.

**Thin sectioning.** Bacterial cells were prefixed with a 1.5% solution of glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h. After washing thrice with the same buffer, the cells were refixed with a 1% solution of $OsO_4$ in the buffer at 20°C for 3 h and then dehydrated. The specimen was embedded in Spurr epoxy resin and cut into thin sections. The sections were mounted on Formvar-coated grids and contrasted with a 3% solution of uranyl acetate in 70% ethanol for 30 min and then additionally with lead citrate by the Reynolds method.

## RESULTS

The replicas of freeze-fractured *An. polyendosporus* cells exhibited the presence of a convex inner leaflet (the so-called PF face) and a concave outer leaflet (called EF face) of the cytoplasmic membrane (Figs. 1–8). The PF face contained a great number of common intramembrane particles (IMPs) 9 to 12 nm in size, whereas the number of such particles on the EF face was considerably lower, which is typical of the membranes of many bacteria [9]. The distribution of IMPs over the faces was not uniform: there were numerous smooth zones on these faces, often having the form of round plaques (Fig. 2) or polygonal patches and strips (Figs. 1 and 5), where IMPs were scarce. These zones commonly had two or more layers. For this reason, these intramembrane structures were named intramembrane lamellar structures (ILSs). Correspondingly, the replicas of freeze-fractured membranes exhibited not only the PF face (Fig. 3) or EF face (Fig. 4) but also other layers, 40 to 50 Å thick, overlying the faces. Such a location of these layers on replicas suggests that in situ they are located in the hydrophobic interior between the outer and inner CM leaflets. The freeze-fractured ILS shown in Fig. 6 had three layers overlying the E face, which evidently resulted from shifting the fracture plane from one membrane leaflet to another (Figs. 3–6). Generally, ILSs could be observed on both faces of the plasma membrane and were 20 to 30 nm wide and 2 to 3 μm long. Unlike the PF and EF faces of the cytoplasmic membrane, the ILS leaflets did not have IMPs of usual sizes but had sparse IMPs of larger sizes (about 150 Å in diameter) (Figs. 1, 3, and 5), which presumably represented lipid particles. The ILS layers adjacent to the outer CM layer were almost free of large IMPs (Figs. 4 and 6). Some monolayer ILSs had a vesicular appearance, as if being composed of numerous adjoining hemispheres (Fig. 7). ILSs were observed in 20–30% of *An. polyendosporus* cells grown on potato agar or in a synthetic medium to different growth phases (Figs. 1–8). The predominant cellular location of ILSs was subcentral, although they might occur in different parts of the cell. Extended, sometimes multilamellar, ILSs were most frequent in bacterial cells taken from lawns on agar media and kept aerobically at 5°C for 2 to 24 h. Most of the cells were viable and up to 60–80% of them contained ILSs.

Taking into account that biomembranes are fractured along their hydrophobic interior, it can be assumed that multilamellar intramembrane structures are generally composed of three flat bilayer lamellae built in the CM as shown in Fig. 6.

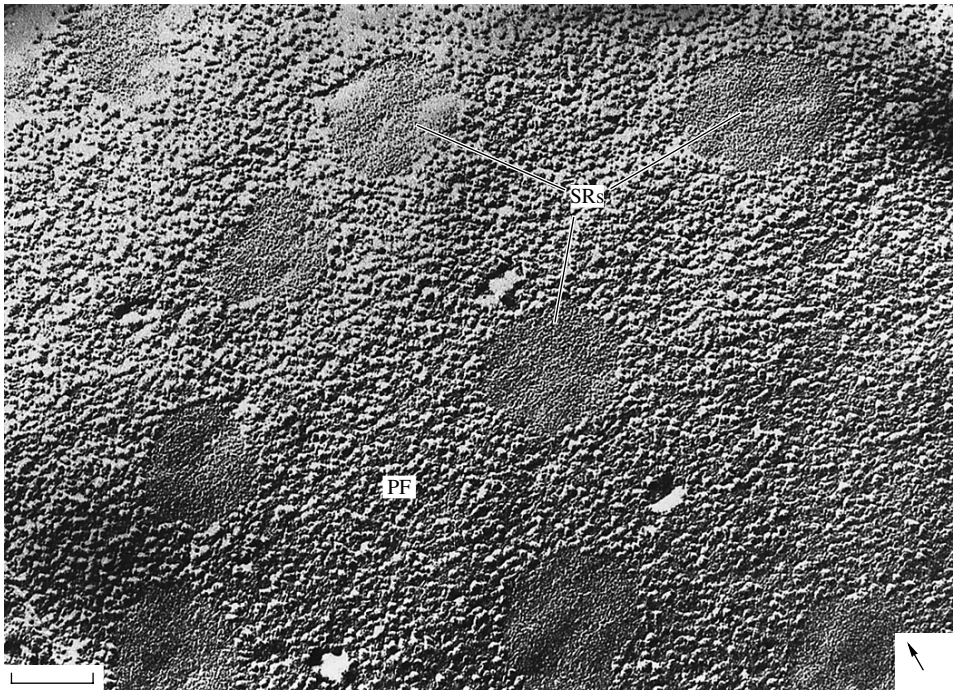


**Fig. 2.** Freeze-fracture replica (PF face) of the CM of *An. polyendosporus* strain PS-1 grown on potato agar. The round smooth regions of the CM contain no IMPs. SR signifies smooth region. Bar = 0.1 μm.

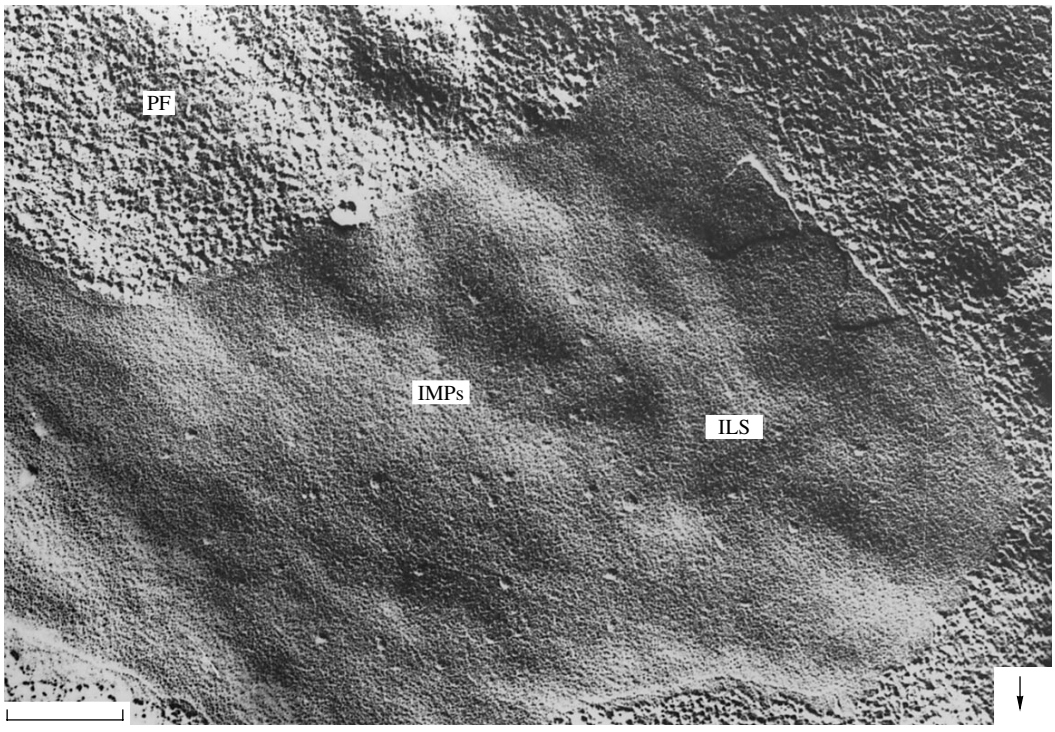


**Fig. 3.** Freeze-fracture replica (PF face) of the CM of *An. polyendosporus* strain PS-1 grown on potato agar. An extended intramembrane lamellar structure (ILS) with large IMPs can be seen. Bar = 0.1 μm.

The analysis of the thin sections of *An. polyendosporus* cells prepared by the standard technique showed that this bacterium lacks intracytoplasmic membrane structures, such as mesosomes, and that the cytoplasmic membrane of this bacterium does not have a typical three-layer appearance on thin sections. This made difficult the observation of ILSs on thin sections, except that ILSs could easily be seen on the thin sec-

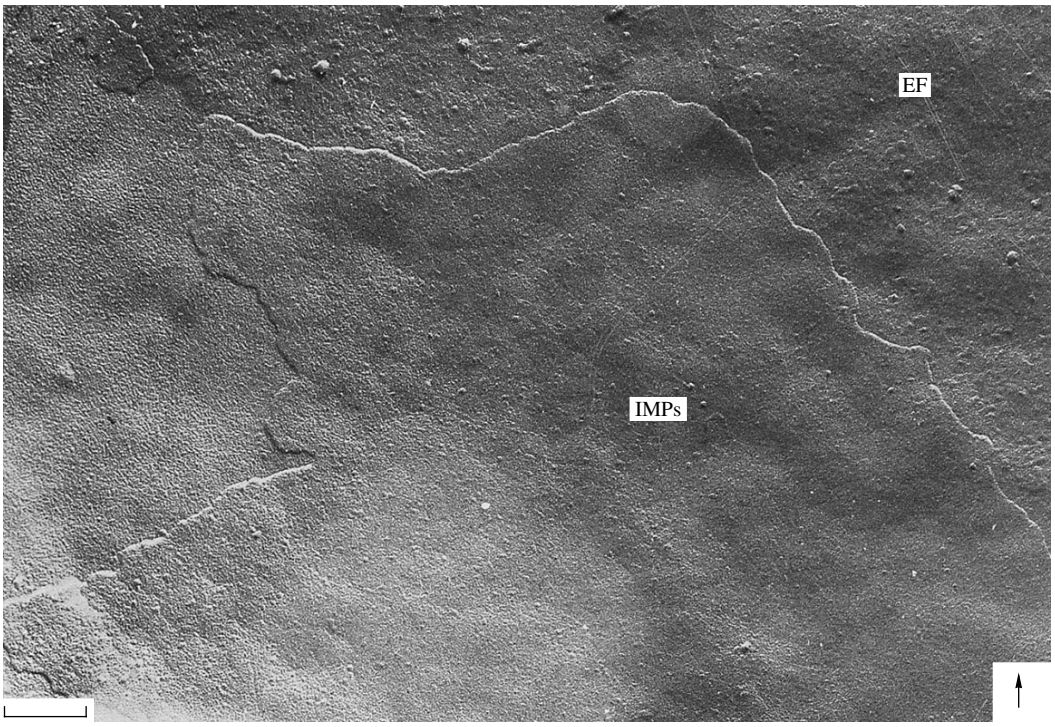


**Fig. 4.** Freeze-fracture replica (EF face) of the CM of *An. polyendosporus* strain PS-1 grown on potato agar. An extended ILS with sparse IMPs can be seen. Bar = 0.1 μm.

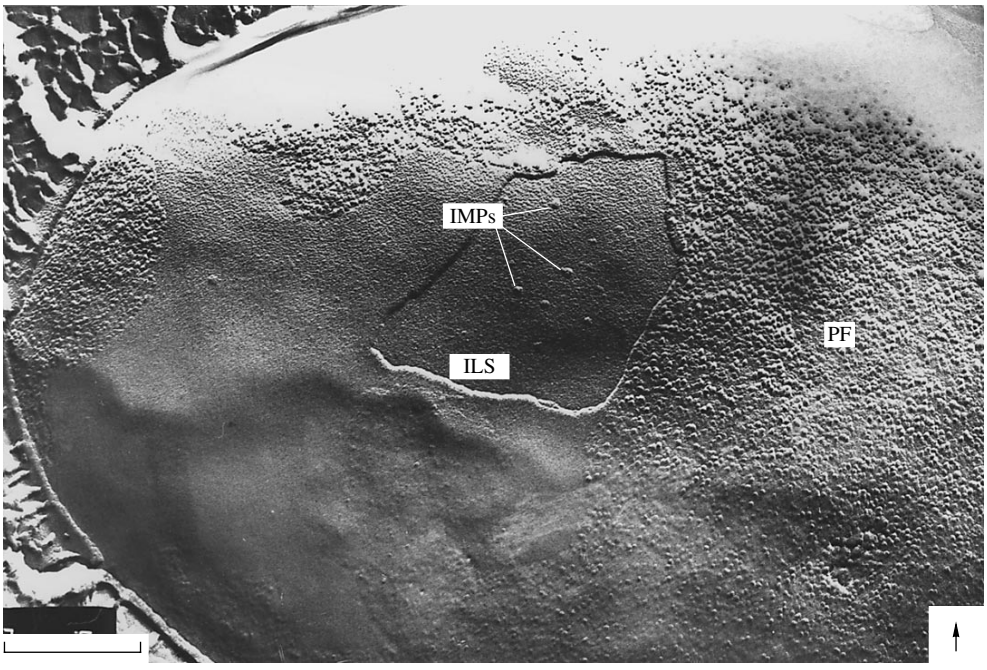


**Fig. 5.** Freeze-fracture replica (PF face) of the CM of *An. polyendosporus* strain PS-1 grown on potato agar. A fragment of an ILS located below the PF face can be seen. Bar = 0.1 μm.

tions of some oval cells (Fig. 9). Further analysis showed that, unlike the cytoplasmic membrane of young cells, the CM of the rod-shaped and polygonal *An. polyendosporus* cells (Fig. 10) from senescent cultures (4–5 days of cultivation in the presence of 0.8% glucose) had a distinct three-layer appearance. The periplasmic space of most of those cells showed the presence of extended regions with a normal three-layer membrane (Fig. 11), which was presumably formed through the evagination and growth of the outer CM

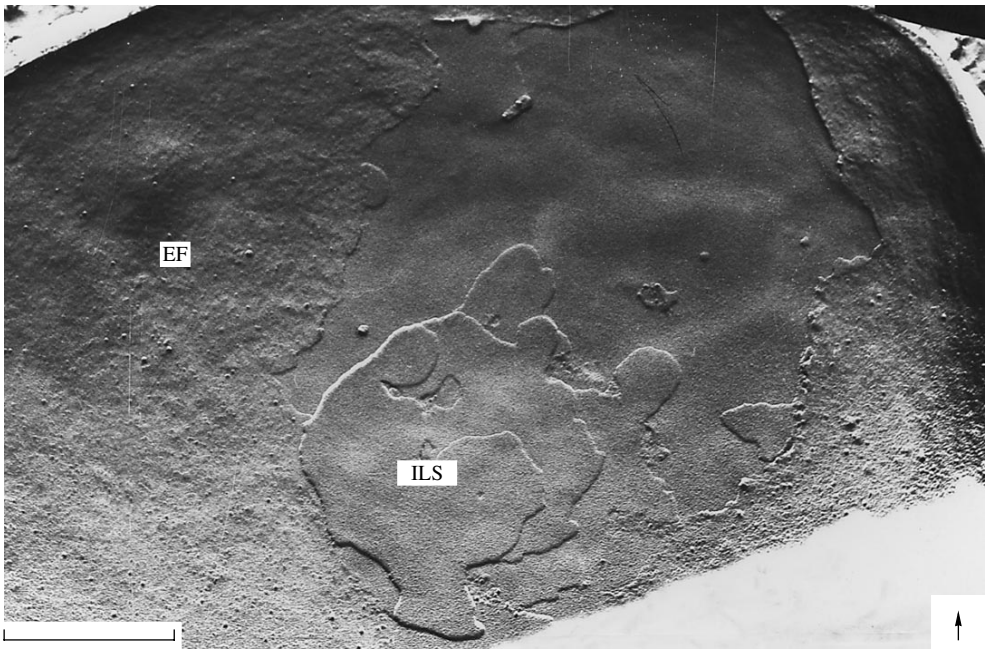


**Fig. 6.** Freeze-fracture replica (EF face) of the CM of *An. polyendosporus* strain PS-1 grown on potato agar. A polar fragment of a multilamellar ILS without IMPs is seen. Bar = 0.1 μm.

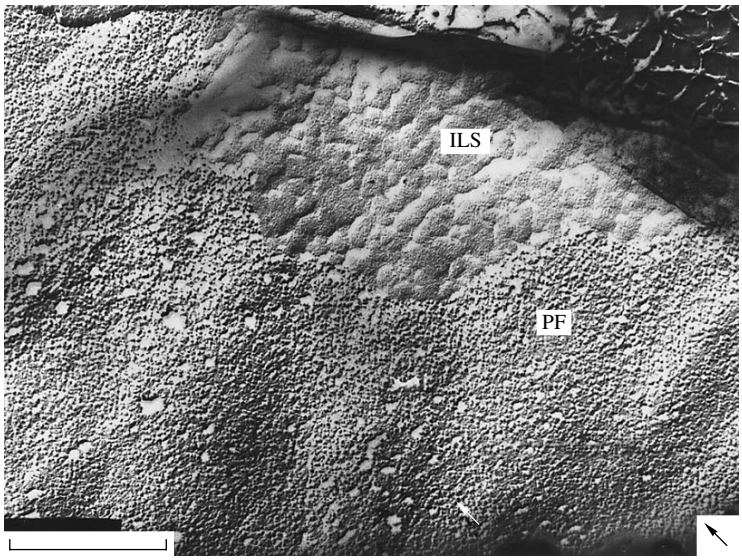


**Fig. 7.** Freeze-fracture replica (PF face) of the CM of *An. polyendosporus* strain PS-1 grown on potato agar. ILS with a vesicular structure is seen. Bar = 0.1 μm.

leaflet. A similar process was described for the bacterium *S. thermosulfidooxidans* [6].

In phylogenetic and physiological properties, the bacterium *An. polyendosporus* is close to saccharolytic clostridia [5]. In light of this, we attempted to reveal ILSs in the saccharolytic *Clostridium* sp. strain 15 and found that some cells of this bacterium contained ILSs in the form of 0.5 μm wide and 1 μm long patches composed of closely contacting and probably fused vesicles 150–300 Å in diameter (Fig. 12).

## DISCUSSION

The possibility of the existence of flat inverted lipid membranes was first revealed in experiments with artificial membranes [2, 10]. In this paper, we present electron microscopic evidence that ILSs can also be

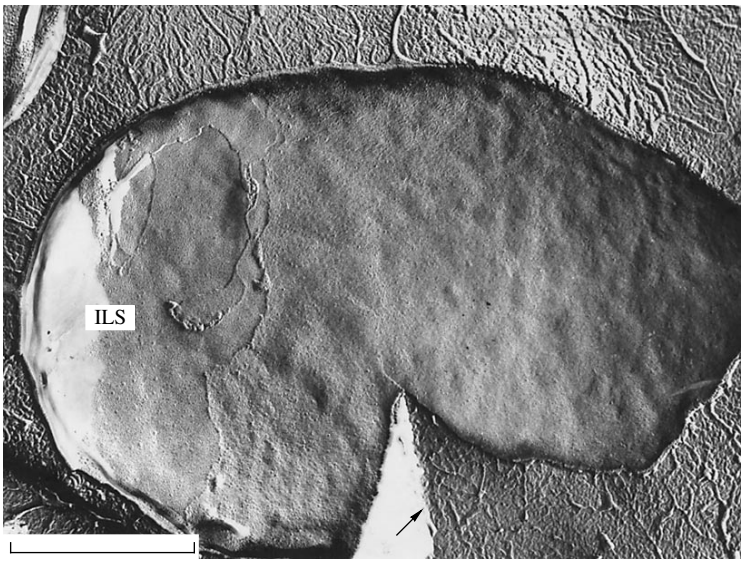


**Fig. 8.** Freeze-fracture replica (EF face) of the CM of *An. polyendosporus* strain PS-1 grown in a chemically defined medium with glucose. Bar = 0.1 μm.

formed in the membranes of bacterial cells grown in optimal nutrient media at normal growth temperatures and pH values. The evidence was obtained from the analysis of freeze-fracture replicas and thin sections prepared by conventional methods and by the freeze-substitution technique [11]. The location of ILSs in the hydrophobic interior of the CM follows from the occurrence of these structures on both P and E faces of freeze-fracture replicas and from the specific appearance of ILSs on thin sections, where they look like inverted lipid membranes bounded by the monolayer leaflets of the CM. Analysis of the structural organization of ILSs showed that (1) ILSs may be multilamellar and have smooth leaflets of different lengths and shapes; (2) they distinctly differ from IMPs commonly present in the CM; (3) paired membranes may have the form of thylakoids; (4) in cross thin sections, the peripheral regions of the CM are bifurcate [11]; and (5) the ILSs of sulfobacilli have a corrugated appearance [6].

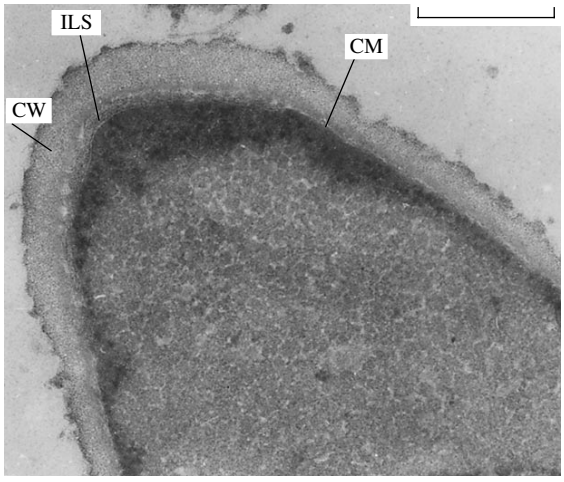


**Fig. 9.** Thin section of a vegetative cell of *An. polyendosporus* with an ILS. CM, cytoplasmic membrane; CW, cell wall. Bar = 0.1 μm.

The data presented in the given paper and those published earlier [6, 11] allowed us to schematically represent several putative mechanisms of the ILS formation (Fig. 13). For simplicity, the drawings in Fig. 13 show only the arrangement of lipid layers and do not show membrane protein particles or IMPs. In some regions of the CM, the common lipid bilayer undergoes rearrangements that give rise to ILSs. The suggestion that ILSs may consist of nonpolar lipids is not confirmed by the electron microscopic analysis of thin sections. As is evident from Figs. 9 and 11, the ILS layers are made of two sublayers, one of which is electron-transparent and obviously hydrophobic and the other is electron-opaque and obviously hydrophilic. Such an appearance on electron micrographs is typical of common membranes composed of polar lipids. The analysis of the thin sections of *S. thermosulfidooxidans* cells [6, 11] showed that the ILSs of this bacterium may represent inverted lipid membranes, such as those depicted in Fig. 13, A–E. Such an arrangement of the ILS layers is confirmed by their cryofractographic analysis (Figs. 1, 3–8, and 12). Inasmuch as membrane bilayers are fractured along their hydrophobic interior, the formation of ILSs shown in Figs. 3–5 is most likely to occur according to schemes A–A' and B–B' in Fig. 13, whereas the ILSs shown in Figs. 1, 6, and 8 are most likely formed

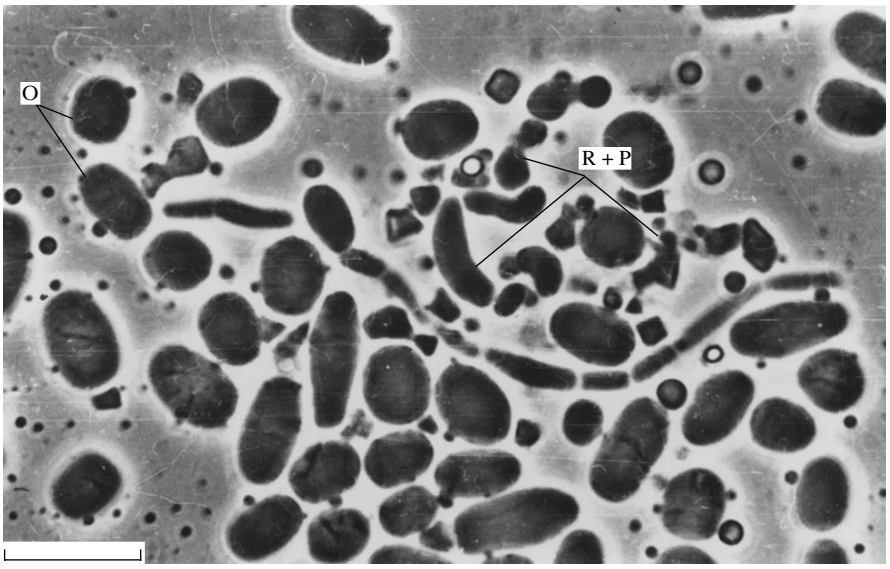


**Fig. 10.** Phase-contrast microscopy of a 4-day-old *An. polyendosporus* surface culture grown on potato agar with 0.8% glucose. O, oval cells; R + P, rod-shaped and polygonal cells. Bar = 5 μm.

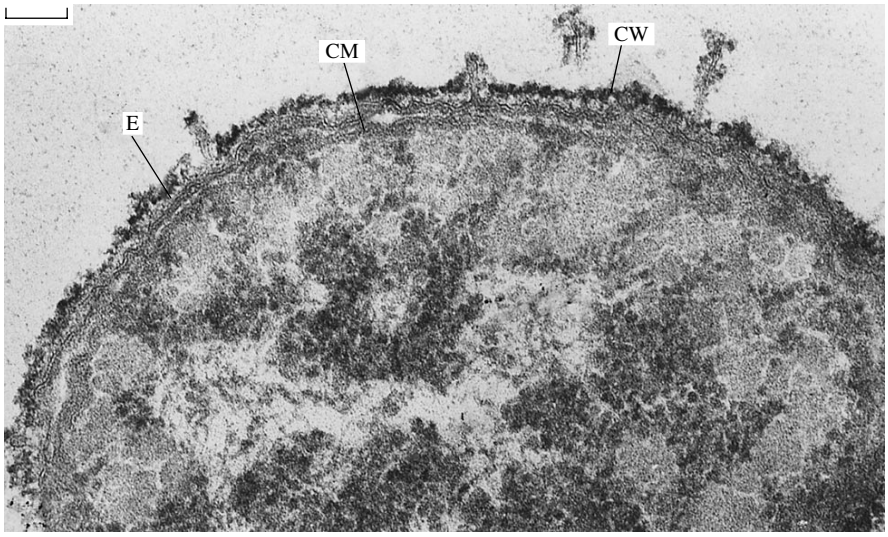


**Fig. 11.** Thin section of a 4-day-old *An. polyendosporus* rod-shaped cell grown on potato agar with 0.8% glucose. E, evaginate. Bar = 0.1 μm.

according to schemes D–D'. The ILSs shown in Figs. 7 and 12 are presumably formed according to schemes E–E'.

ILSs can be produced either by the invagination of the outer CM leaflet [11] with the formation of a thylakoid structure (this mechanism of ILS formation is shown in Figs. 13A, 13C, and 13D) or by the fusion of intramembrane lipid vesicles, as follows from the analysis of the electron micrographs presented in Figs. 7 and 12 (this mechanism of ILS formation is shown in Fig. 13, E–E'). The mechanism of ILS formation through the fusion of inverted lipid vesicles has also been postulated for artificial lipid membranes [2, 10], although such membranes substantially differ from natural biomembranes in chemical composition and physicochemical properties. The formation of lipid vesicles inside the lipid bilayer of natural biomembranes may occur through the CM fusion near the growing septa of dividing cells and through the local accumulation of newly synthesized lipids in some regions of the CM.

The evagination of the outer CM leaflet toward the periplasm (Fig. 13, B) may be due to the differential distribution of intramembrane lateral pressure, which

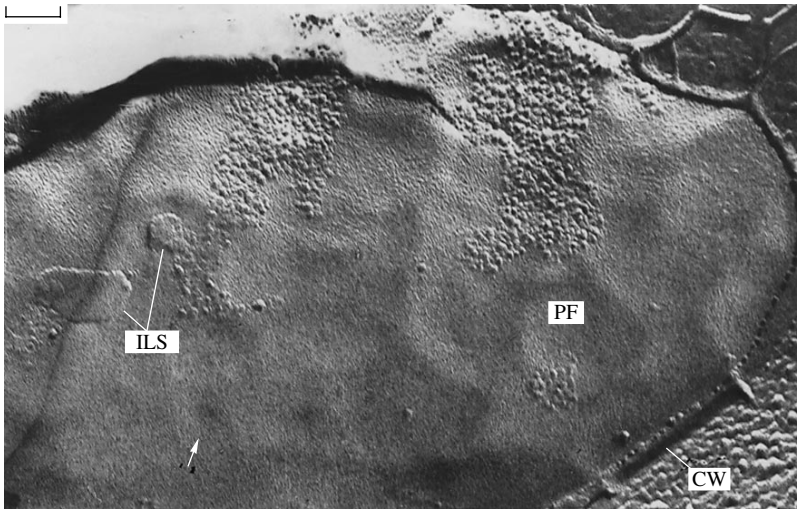


**Fig. 12.** Electron micrograph of the P face of the CM of *Clostridium* sp. strain 15 with an ILS resembling merged vesicles. Bar = 0.1 μm.

has been reported to depend on the composition of the lipid bilayer [12]. The outer CM leaflet seems to evaginate easier than the inner leaflet can invaginate, since the latter contains more IMPs and is bound to ribosomes, the nucleoplasm, and, as has recently been shown [13], the bacterial cytoskeleton. The lipid asymmetry of bacterial membranes should also be taken into account [14, 15]. The evagination of the outer CM layer is of interest in relation to the biogenesis of the outer membrane of the cell wall (or second lipoprotein membrane) of some gram-negative bacteria, such as the recently isolated spore-forming bacteria *Sporomusa* [16] and *Sporohalobacter* [17]. Taking into account that spores have no cell wall (CW), the outer membrane of vegetative cells must rapidly form during the outgrowth of spores. If this is the case, it would be reasonable to suggest that the outer membrane of the CW is formed through the evagination and expansion of the outer leaflet of the sporal CM.

The reason for the corrugated appearance of the *S. thermosulfidooxidans* ILSs is still unclear. Presumably, this may be related to the specific composition of the membrane lipids of this bacterium. Indeed, as shown by Tsaplina *et al.* [18], the membrane lipids of *S. thermosulfidooxidans* VKM B1269 contain up to 60–70% of ω-cyclohexane acid. At the same time, the major fatty acids of the *An. polyendosporus* membranes, like those of the butyric acid clostridia, are myristic ($C_{14:0}$), palmitic ($C_{16:0}$), and hexadecenoic ($C_{16:1}$) acids (unpublished data).

The general functions of ILSs are probably the same as those of the nonbilayer regions of biomembranes [2–4, 11]: they may represent a depot of lipids and whole membrane units, which are utilized during the active growth of cells or when it is necessary to rapidly enlarge the CM, to repair it, or to form the mesosome-type intracytoplasmic membrane structures. Alternatively, ILSs may be involved in the translocation of lipids across the membrane and in the processes related to cell growth and differentiation, such as the separation of nucleoids, formation of septa, and sporogenesis.

Some functions of ILSs may be species-specific. For instance, ILSs may be responsible for the tolerance of the obligately anaerobic bacterium *An. polyendosporus* to oxygen: the vegetative cells of this bacterium incubated on solid nutrient media in the presence of atmospheric oxygen remain viable for as long as 3–7 days. After 1–3 days of such incubation, the number of ILSs and their mean size considerably increased (Fig. 6). The additional lipid layers of ILSs may reduce the CM permeability to oxygen. As for the effect of the lipid composition of membranes on their permeability to oxygen, it has been demonstrated by Subczynki *et al.* [19]. In cyanobacteria, the surface structures formed by heterocysts considerably decrease the penetration of oxygen into cells [20]. The functional role of ILSs in microorganisms is to be investigated in depth.

Flat ILSs and intramembrane lipid vesicles represent specific compartments within the CM, whose existence is indicative of complex compartmentalization processes in biomembranes. The formation of such membrane structures had been predicted by Cullis *et al.* [2] during the investigation of artificial lipid membranes. The presence of ILSs in such dissimilar microorganisms as anaerobic heterotrophic mesophilic bacteria and aerobic mixotrophic thermoacidophilic bacteria suggests that they are widespread among many microorganisms.

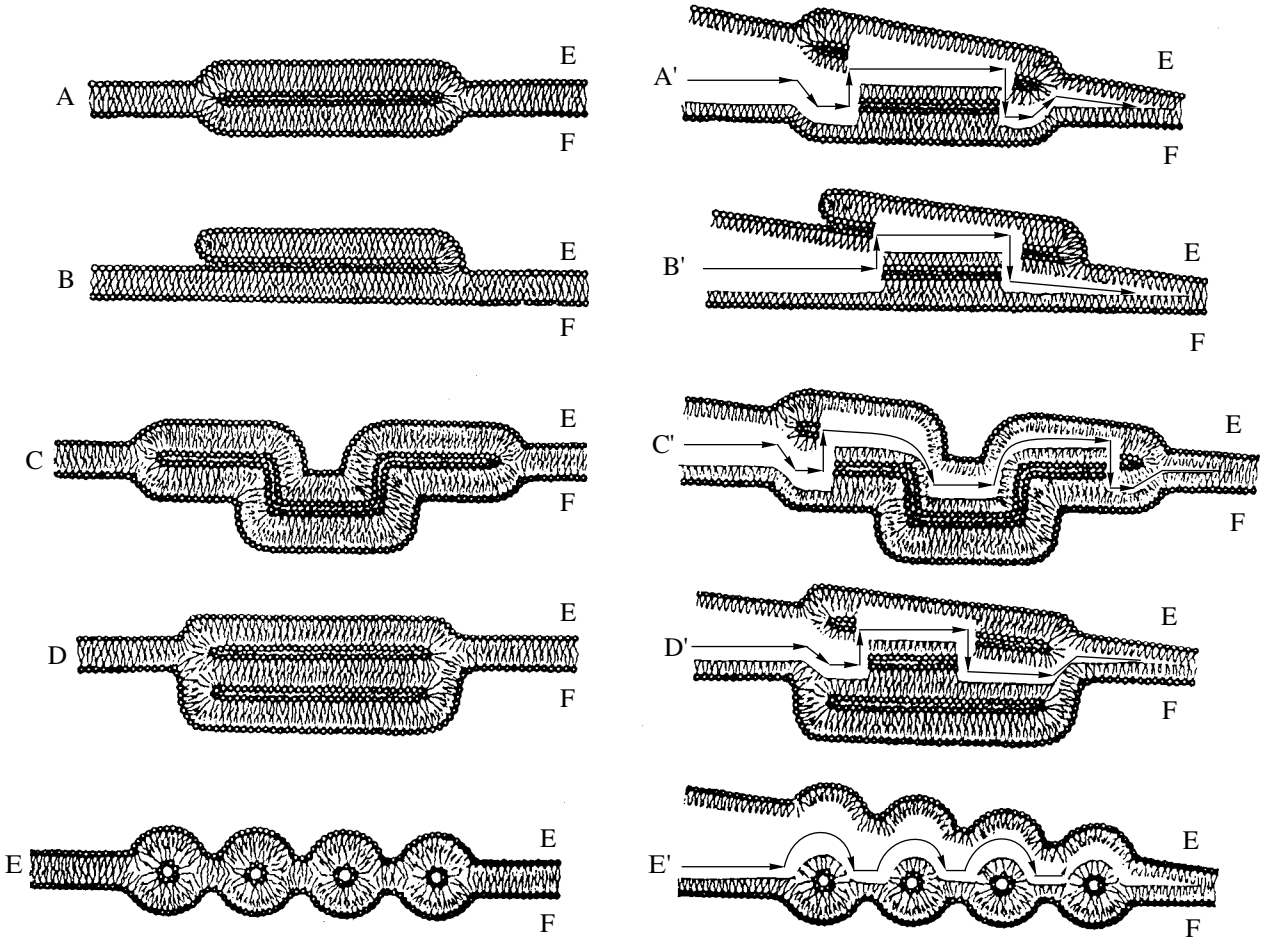


**Fig. 13.** Schematic representation of the arrangement of lipid molecules in the CM and ILSs deduced from electron microscopic data. A–D represent flattened ILSs with smooth surfaces, either trilamellar (A–C) or multilamellar (D). B illustrates the evagination of the outer CM leaflet toward the periplasmic space. C illustrates ILS with a corrugated appearance typical of *Sulfobacillus thermosulfidooxidans* [6, 11]. E illustrates lipid vesicles in the hydrophobic interior of the CM. E denotes the outer (periplasmic) side of the CM. F denotes the inner (cytoplasmic) side of the CM. Drawings A'–E' show the likely directions of the fracture of CM, ILSs, and lipid vesicles.

## ACKNOWLEDGMENT

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