Membrane Ultrastructure of Alkaliphilic Bacillus Species Studied by Rapid-Freeze Electron Microscopy

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Cells of Bacillus firmus OF4 and Bacillus alcalophilus were examined by rapid-freeze freeze-fracture and freeze-substitution electron microscopy. No special vesicular structures linked to growth at alkaline pH were found, either within or associated with the cytoplasmic membrane. The cytoplasmic membranes of the alkaliphilic bacilli and the neutrophilic Bacillus subtilis BD99 were indistinguishable. Distinctive intramembrane particle rings, presumed to be flagellar structures on the basis of distribution and morphological characteristics, were found in all of these species. These observations indicate that the adaptations required to effect oxidative phosphorylation and flagellar rotation at extreme alkaline pH occur without gross morphological rearrangement.

The cytoplasmic membranes of the alkaliphilic bacteria contain machinery to couple the chemiosmotic energy of transmembrane ion gradients to diverse work functions. A unique problem arises at extremely alkaline values of the growth pH, since the much smaller cytoplasmic pH results in the bulk electrochemical gradient being much smaller than that generally found in prokaryotes or in some of the same strains grown at lower pH (8). While this energetically adverse gradient is bypassed by coupling flagellar rotation (5, 19) and solute symporters (14) to sodium ions rather than protons, the F1F0 ATP synthase remains proton coupled (4, 6, 9).

Conceivably, adaptation for energy coupling at alkaline pH could involve changes at the level of ultrastructure. For example, the utilization of sodium ions rather than protons by the flagellar motor could involve morphologically distinct structures. With respect to proton-coupled oxidative phosphorylation, the ATP synthase and respiratory chain components might be protected from the extremely alkaline external pH by being sequestered within special cytoplasmic or membrane-associated vesicular structures. Membranous cytoplasmic organelles that could play such a role were not found in Epon-embedded thin sections of the alkaliphilic bacilli (13). As regards clustering of the oxidative phosphorylation machinery, the ATP synthase was found to be distributed all over the membrane of Bacillus firmus RAB in low-temperature Lowicryl-embedded immunolabeled thin sections (17). Nevertheless, special membrane structures, envisioned as enclosed bulges that are continuous with the cytoplasmic membrane, have recently been proposed as a solution to the energetic problem of oxidative phosphorylation in alkaliphiles (1, 18). Thus, to further explore the possibility of structural correlates to alkaliphily, we have used rapid freezing to capture the in situ distribution of membrane proteins and compare the membrane ultrastructure of facultatively alkaliphilic B. firmus OF4, which can be grown at both pH 7.5 and pH 10.5, with that of the obligately alkaliphilic Bacillus alcalophilus (ATCC 27467) and the neutrophilic Bacillus subtilis BD99.

B. firmus OF4 was grown at both pH 7.5 and pH 10.5, B. subtilis BD99 was grown at pH 7.5, and B. alcalophilus was grown at pH 10.5 (8). The bacteria were grown in batch cultures and harvested in the logarithmic phase of growth at a stage when the pH of the medium had changed no more than 0.2 pH units from the initial value. Highly buffered, malate-containing media, described previously (8), were used. The cells were checked for motility and concentrated 100-fold by centrifugation and resuspension in growth medium. Samples (10 μl) of the concentrated suspensions were applied onto gelatin platforms on metal planchette and slam frozen without delay on a liquid helium-cooled copper block of a Life Cell model CF100 rapid-freezing machine. For freeze-fracture freeze-etch replication, the specimens were knife fractured at −105°C in a Cressington CF50 freeze-fracture apparatus. The samples were replicated directly after fracture or after being etched for 10 min under a vacuum of better than 10−7 torr (1 torr = 133.322 Pa). The samples were rotary shadowed at a 45° angle with platinum and then rotary coated with carbon. The replicas were cleaned as described previously (11). For freeze-substitution, the samples were substituted in 1% osmium tetroxide acetone, stained with uranyl acetate and hafnium chloride, and embedded in Araldite as described previously (12). The freeze-etch replicated and freeze-substituted thin sections were viewed at 80 kV on a JEOL 100CX electron microscope. Criteria detailed previously (12) were used to select areas with negligible ice crystal damage for analysis.

The P-fracture face (11) of the lipid bilayer, in the two alkaliphilic Bacillus species, had a smooth surface studied...
with intramembrane particles (Fig. 1). Over 100 μm² of freeze-fractured P-face membranes was examined for each species and condition. There was no observable difference between pH 10.5- and pH 7.5-grown B. firmus OF4 preparations and between the alkaliphiles and the B. subtilis control. The preparations had a mean density of about 5 × 10³ particles per P.μm² of P-fracture face area. It is commonly held that the intramembrane particles visualized in freeze-fracture replicas correspond to integral membrane protein or protein complexes (16). Immunolabeling had previously indicated a global distribution of the F₁F₀ ATP synthase in the membranes of the alkalophilic bacilli. We found no hint of a corresponding global distribution of bulges or pits that might be indicative of enclosed membranous spaces of size sufficient to harbor assemblies of whole respiratory chains and the ATP synthase as proposed by others (1, 18).

Flagellar intramembrane particle rings are distinctive of the cytoplasmic membranes of flagellated bacteria (Aquaspirillum serpens [3], Escherichia coli [11], Streptococcus sp. [11], and Salmonella typhimurium [12]). We observed intramembrane rings morphologically analogous to such
flagellar structures in all three Bacillus species used in this study (Fig. 1b to d). The defining characteristic of the flagellar rings is a large central knob, about 10 nm, surrounded by a ring of particles of uniform size, about 6 to 7 nm; the radial spacing between central knob and ring particle is about 20 to 25 nm. The central feature is thought to be the proximal rod segment of the flagellum, and the ring particles are thought to be the ion channels responsible for its energization (10). We focused further study on B. firmus OF4 (Fig. 2a). When the sample was etched before replication, the central core of these structures collapsed. In electron micrographs that were reversed so that the metal appeared white, the depressed area surrounding the central knob (that resulted from the etching) appeared dark (compare Fig. 2b to d with Fig. 2a), indicating absence of metal and material. Previous work had shown such etch-dependent change to be a characteristic of flagellar particle rings (11, 12). This property made the particle rings readily detectable in etched replicas, which therefore were used to assess their distribution. For pH 10.5- and pH 7.5-grown B. firmus OF4, 33 and 27 ring structures, respectively, were found in 100 μm² of P-face membrane. Measurements on pH 10.5-grown bacteria showed that the rings had mean outer and inner diameters of 32.7 ± 1.9 and 23.4 ± 1.7 nm, respectively (Fig. 2). The numbers of particles per ring were 9.3 ± 1.4 (n = 20) for B. firmus OF4 cells grown at pH 10.5 and 9.0 ± 1.9 (n = 20) for cells grown at pH 7.5. We did not observe rings with more than 12 particles. We cannot prove that these rings are flagellar structures, since motility mutants are not yet available for the alkaliphilic Bacillus species. However, the dimensions of the rings, their density per unit area of membrane, and the morphological changes effected upon etching offer persuasive evidence, taken together, that this is the case. If each ring particle is an ion channel (10), then the identical ring structures found in both alkaliphilic and neutrophilic Bacillus species together with the pH independence of particle number per ring, indicate that flagellar rotation at alkaline pH does not involve an increased number of channels. Nor, apparently, are vesicular membrane specializations involved, since the particle rings were not found atop bumps or depressions as is the case, for example, for secretory fusion rosettes (2). These observations, then, are consistent with the suggestion (15), made earlier on the basis of physiological data, that the construction of sodium-powered flagellar motors differs at the molecular rather than ultrastructural level from the construction of proton-powered ones.

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