VISUALIZATION OF PORES (EXPORT SITES) CORRELATED WITH CELLULOSE PRODUCTION IN THE ENVELOPE OF THE GRAM-NEGATIVE BACTERIUM *ACETOBACTER XYLINUM*

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ABSTRACT

The Gram-negative bacterium *Acetobacter xylinum* assembles a cellulose ribbon composed of a number of microfibrils in the longitudinal axis of its envelope. The zone of ribbon assembly was investigated by freeze-etch electron microscopy. Freeze-etching revealed, beneath the cellulose ribbons, a linear array of pores on the lipopolysaccharide membrane. These pores have a rim diameter of 120–150 Å and a central hole or deepening of ~35 Å. The axes of pore arrays closely coincide with linear arrays of 100 Å particles on the E- and P-faces of the fractured lipopolysaccharide membranes. Pores and particles in the lipopolysaccharide membrane are probably congruent. The pores are hypothesized to be the export sites (penetration sites) for cellulose.

KEY WORDS *Acetobacter xylinum* · cellulose · Gram negative bacterium · pores · lipopolysaccharide membrane

Cellulose is the most abundant polymer in green plants and can be found in certain fungi, protozoa, and procaryotes. The biogenesis of cellulose initially poses two major problems: the biosynthesis of linear β-1,4 glucan chains and the (biological) mechanism of their crystallization to form cellulose microfibrils. So far, neither of these important problems has been resolved. Over decades now, one of the most famous model organisms for studying cellulose biogenesis is the Gram-negative bacterium *Acetobacter xylinum*. Until recently, it was generally believed that the synthesis of crystalline microfibrils by *Acetobacter* is an extracellular process. The work of Brown and co-workers (2) and Zaar (18) has now shown that cellulose is produced in close contact with the bacterial envelope. Each bacterium assembles on its surface one flat ribbon consisting of cellulose. These ribbons are composed of a varying number of cellulose microfibrils with an average diameter of 10–20 to 30–40 Å (18). The microfibrils lie side by side in a horizontal axis, thus giving rise to the flat ribbon-like structures. Most ribbons are twisted in a very regular manner (2, 18). Ribbons reaching more than 10 μm in length project over one pole of the rod-like bacteria and taper to a point at the end distant from the cells. The assembly of the cellulose ribbons seems to be restricted to only a very small area in the longitudinal axis of the bacterial envelope (2, 18). Furthermore, each microfibril of a ribbon is suggested to have an independent terminus in the bacterial envelope (2, 18).

This is a report concerning the ultrastructure of the particular area of ribbon assembly in the bacterial envelope as revealed by freeze-etch preparations.

MATERIALS AND METHODS

Culture conditions, harvesting, and cellulose synthesis of cellulose-producing *A. xylinum* (strain ATCC 10 245) were performed as described (18). For freeze-etch preparations, active cellulose-producing bacteria were either fixed for 30 min with 2% glutaraldehyde or left unfixed. Fixed specimens were washed before stepwise transfer into 25% glycerol. Unfixed as well as fixed bacteria were frozen on cardboard disks in Freon 22 cooled by liquid nitrogen (17). Fracturing, etching (1 min at −100°C)

and replicating were performed on a Balzers Model BA 360 (Balzers AG für Hochvakuumtechnik, FL-9496 Balzers, Fürstentum, Liechtenstein) (10).

RESULTS

Acetobacter is a Gram-negative rod-like bacterium with the well known envelope structure composed of an outer lipopolysaccharide membrane, an intermediate peptidoglycan layer, and the plasma membrane. Freeze-fracture planes in *A. xylinum*, as in other Gram-negative bacteria, occurred most frequently in the lipopolysaccharide membrane. The E-fracture faces (EF) (for freeze-etch nomenclature, see reference 1) of these membranes are studded with 50–60 Å particles randomly arranged (Fig. 1). In other Gram-negative bacteria, the majority of these particles is thought to consist of protein (15) or the lipopolysaccharide-protein complexes of the outer membrane (16).

A conspicuous feature of the lipopolysaccharide membrane fracture faces of cellulose-producing *A. xylinum* cells is a long row of large particles. These particles occurred on both the E-fracture (EF) and P-fracture faces (PF) (Figs. 1, 2, 3, and 4) of the membrane. The rows extend, in the longitudinal axis of the bacterial rods, most of the length of the bacteria. In general, these particles are spherical on the E-fracture face with an average diameter of 100 Å. However, also short rod-like structures could be seen on the E- and particularly on the P-fracture face (Figs. 1, 2, and 4). Sometimes, the appearance of these rod-like structures suggested plastic deformation. During cell fission, a row of particles is divided perpendicular to its long axis, and the two particle arrays are partitioned to the daughter cells. In cases where cellulose ribbons still lay on the etched surface of the lipopolysaccharide membrane (ES), the axes of the microfibrillar ribbons and the particle rows on E- and P-fracture faces closely coincided (Fig. 2).

Partial or total detachment of the cellulose ribbons revealed, beneath each ribbon, on the E-surface (ES) of the lipopolysaccharide membrane a row of distinct pits (Figs. 2, 3, 4). Furthermore, fractures unveiling E-surfaces (ES) and P-fracture faces (PF) at the same time showed that the row of pits on the bacterial surface extends in the longitudinal axis of the cells in exact alignment with the particle rows within the membrane (Figs. 3 and 4).

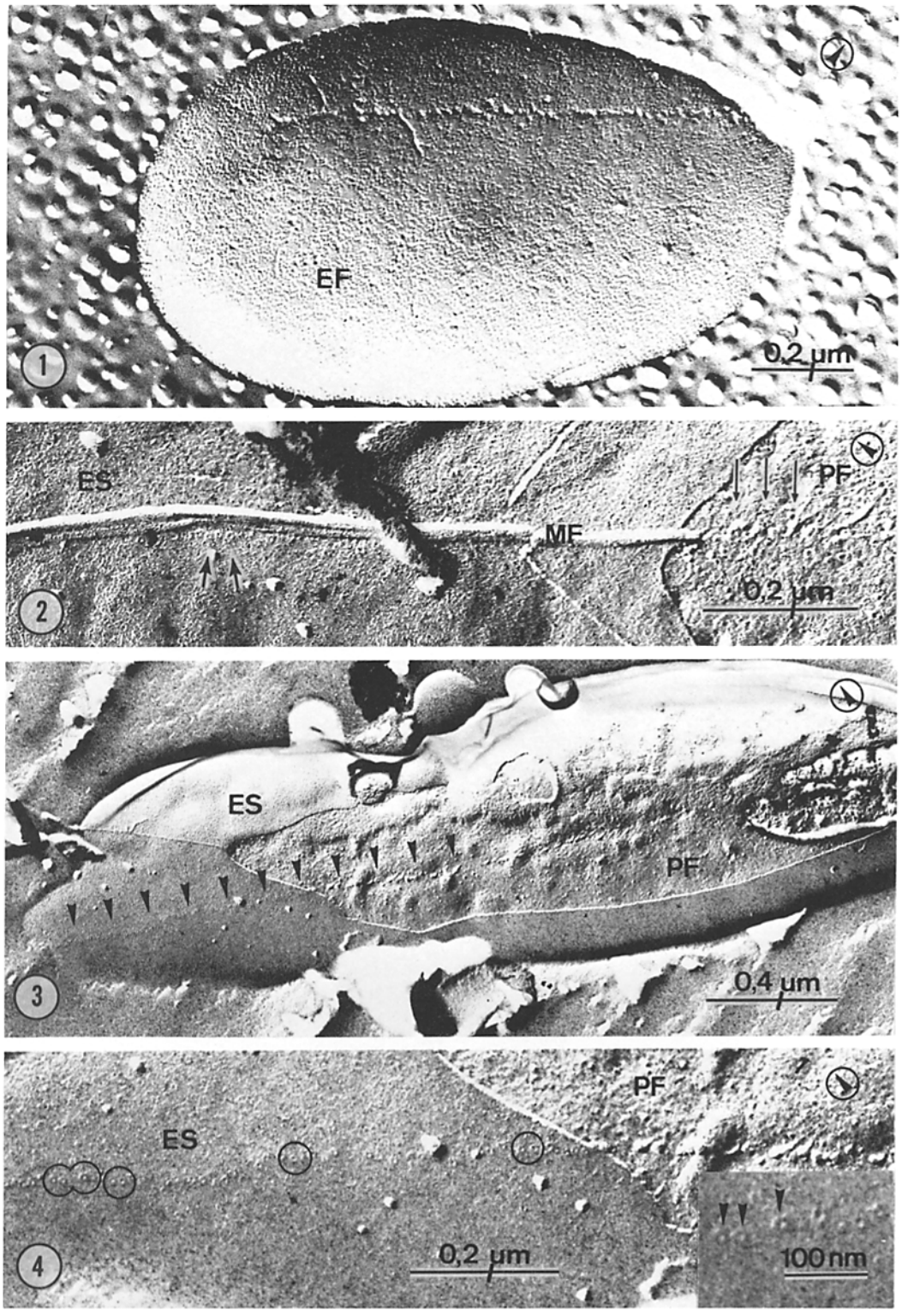
These pits have a crater-like ring wall structure with an average diameter of 120–150 Å and a central deepening or hole ~35 Å in diameter (Fig. 4). It is suggested that these pits are pores in the lipopolysaccharide membrane with a central opening of ~35 Å. The particles and pores per unit length were almost identical in frequency (average 3.8 pores/particles per 100 nm). Therefore, each pore on the surface probably corresponds to a 100 Å particle within the bacterial envelope. The number of particles varied between 12 in each of

FIGURE 1 The E-fracture face (*EF*) of the lipopolysaccharide membrane of active cellulose-producing *Acetobacter xylinum* shows a random distribution of small particles 50–60 Å in diameter. Large particles ~100 Å in diameter are arranged in a distinct row in the longitudinal axis of the bacterial rods. Bacteria were fixed with glutaraldehyde and treated with glycerol. Encircled arrowheads indicate the direction of shadowing. Bar, 0.2 μm.

FIGURE 2 A microfibrillar cellulose ribbon (*MF*) lying on the etched surface (*ES*) of a bacterium. In cases where the ribbon is drawn away from its original position, pores may be seen beneath (large arrows). On the P-fracture face (*PF*) of the lipopolysaccharide membrane a row of large particles extends beneath the original position of the cellulose ribbon, demonstrating a linear relationship (small arrows). See also Figs. 3 and 4. Unfixed bacterium. Bar, 0.2 μm.

FIGURE 3 Survey view of an etched cellulose-producing *A. xylinum* cell. The cellulose ribbon is totally torn away. On the P-fracture face (*PF*), a linear row of particles extends. This row is continued on the E-surface (*ES*) (outer surface of the bacterial envelope) by a linearly arranged row of pores lying obviously exactly above the particles. See also Fig. 4. Unfixed bacterium. Bar, 0.4 μm.

FIGURE 4 Enlarged portion of Fig. 3 demonstrating the linear relationship between the row of particles on the P-fracture face (*PF*) and the row of pores on the E-surface (*ES*). The pores are crater-like ring wall structures 120–150 Å in diameter with a central deepening or hole of ~35 Å (circles). Unfixed bacterium. Bar, 0.2 μm. (*inset*) Pores from the left hand corner of Fig. 4 at higher magnification. Bar, 100 nm.


EF
0,2 µm
1
ES
PF
MF
0,2 µm
2
ES
PF
0,4 µm
3
PF
ES
0,2 µm
100 nm
4

two dividing cells and 70 in elongating non-dividing cells. A calculation of the membrane area endowed with pores and corresponding particles revealed that only ~0.5% of the total envelope may be engaged in the assembly of cellulose ribbons.

DISCUSSION

Cellulose ribbons produced by *A. xylinum* are assembled on the bacteria (2, 18). Previous work (2, 18) has indicated that each microfibril of a cellulose ribbon probably grows from a site of synthesis or a particular site of export. Each microfibril thus is supposed to have an independent terminus on or in the bacterial envelope. It is reasonable to assume that these sites or termini correspond to the axially arranged row of particles and pores found after freeze-etch preparations in or on the lipopolysaccharide membrane. The correlation of the linear array of particles with the microfibrillar ribbon has recently been discussed by Brown and co-workers (2). Particles (2) and pores are suggested to be associated with the form ng ends of microfibrils in a cellulose ribbon. Cellulose biogenesis in *Acetobacter* seems to be one of the best proven examples of a unidirectional growth of cellulose microfibrils (2, 3, 18). Since biosynthesis and chain elongation of linear polysaccharides generally occurs from one end, the unidirectional growth of microfibrils strongly suggests a parallel organization of extended glucan chains within crystalline microfibrils.

The different frequencies of particles and pores between cells in the course of fission and non-dividing elongating cells probably indicates a growth-dependent extension of the linear microfibril-synthesizing complex. This extension occurs together with an increase in the width of cellulose ribbons (18).

The large particles (100 Å Diam.) found in freeze-etch preparations could represent the multienzyme complexes (see also reference 3) or at least visible parts of these complexes involved in the synthesis of β-1,4 glucan chains and microfibrils. Each particle is thought to be the origin of one microfibril, so the particle must have the capacity to synthesize simultaneously a number of glucan chains. Fractures occurred very rarely in the plasma membrane, so at the moment it is not clear whether these particles span more than the lipopolysaccharide membrane. Biochemical evidence has indicated that the outer envelope is necessary for cellulose synthesis by *Acetobacter* (5). However, enzymes involved in cellulose synthesis occur also in association with the plasma membrane and the periplasmic space (5). Concerning the outer lipopolysaccharide membrane, only phospholipase A has been encountered so far as the major enzymatic activity in Gram-negative bacteria (12, 14).

As an alternative hypothesis, it may be considered that these particles are not multienzyme complexes but represent an accumulation of an intermediate of cellulose (e.g., 4, 8, 9, 13) which, for example, could have been synthesized throughout the bacterial envelope. This intermediate then has to be accumulated in a very specific way in close correlation with the pores.

Of particular interest now are the pores in the lipopolysaccharide membrane. As regards a general function, these pore structures could provide defined pathways through the lipopolysaccharide membrane for hydrophilic macromolecules synthesized within the bacterial envelope. The most simple function of these pores in the case of cellulose could be to provide a channel allowing the completed microfibrils to penetrate the membrane. However, the whole pore structure could, as well, operate as a catalyst promoting the crystallization, e.g., by forcing glucan chains during their immediate synthesis into the form of a microfibril. The pores could thus fulfill the function of an "assemblyase" theoretically proposed by Brown and Willison (3). If a cellulose intermediate exists, the pores should function as "spinning machines" spinning glucan chains into a crystalline microfibril. Here the pores may additionally act through the biochemical transformation of the intermediate to cellulose.

Whatever the exact function of these pores in cellulose biogenesis may be, they are ideal penetration sites for macromolecules. The existence of small hydrophilic pores in the envelope of Gram-negative bacteria seems to be well established now by physiological experiments. These "Porin" channels in the lipopolysaccharide membrane are formed by a particular protein species (11); however, penetration of these pores is restricted to low molecular weight components in solution (6). Recently, Inouyé (7) has proposed, from theoretical considerations, the existence of larger pores in the envelope of Gram-negative bacteria. These kinds of pores should develop by a particular aggregation of a defined number of cylindrical Braun lipoprotein molecules common in the envelope of Gram-negative bacteria (7).

This is the first report on the ultrastructural existence of large, defined pores (penetration sites) in the envelope of a Gram-negative bacterium. Similar large pores could occur in other Gram-negative bacteria but may have been overlooked because of their sparse distribution. In *Acetobacter*, it is their extraordinary arrangement in a linear complex that renders the pores visible.

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