Outer Membrane Protein Pattern of *Eubacterium plautii*

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The outer membrane SDS-PAGE pattern of *Eubacterium plautii* was characterized by a large number of surface exposed low- and high-molecular-mass proteins. Silver stainable carbohydrate was not present. The pattern was clearly distinct from those of outer membrane preparations of *Eubacterium saburreum* and *Fusobacterium nucleatum*. The results are compatible with a Gram-positive cell wall structure in *E. plautii*.

INTRODUCTION

Eubacterium plautii is an anaerobic rod that stains Gram-negative. A morphological description of the organism was given by Plaut (1907). Sèguin (1928), who succeeded in isolating the organism in pure culture, named it *Fusobacterium plauti* (*sic*). Jantzen & Hofstad (1981) found that the fatty acid pattern of the organism differed markedly from those of *Fusobacterium* species. 3-Hydroxy fatty acids, which are invariably present in Gram-negative bacteria as constituents of the cell wall lipopolysaccharide, were absent. Electron microscopical examination showed that the organism had a homogeneous, thick outer layer, not the triple-layered outer membrane characteristic of a Gram-negative bacterium (Hofstad & Aasjord, 1982). Based on these findings, Hofstad & Aasjord (1982) proposed a transfer of the organism from the genus *Fusobacterium* to the genus *Eubacterium* as *Eubacterium plautii* (Sèguin) comb. nov.

The present report deals with SDS-PAGE profiles of outer membrane proteins in *E. plautii* as compared to those of *Fusobacterium nucleatum* and *Eubacterium saburreum*.

METHODS

Bacterial strains and growth conditions. *E. plautii* strains VPI 0310 (= ATCC 29863) and VPI 4145 were obtained from Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Va., USA. *F. nucleatum* Fev 1 was provided by S. E. Mergenhagen, National Institute of Dental Research, Bethesda, Md., USA. *E. saburreum* strain L32 (Hofstad & Skaug, 1978) was isolated from human saliva. The organisms were cultivated in a fluid medium based on tryptone and yeast extract, and supplemented with vitamin B₁₂, haemin, menadion, glucose and (for the cultivation of strain L32) human plasma (Hofstad & Aasjord, 1982). Ascorbic acid was used as the reducing agent. Bacteria were harvested in the late exponential phase of growth.

Preparation of membranes. Outer membrane fractions were prepared as described by Bakken & Jensen (1986). The harvested cells were washed in cold 0.03 M-Tris/HCl, pH 8.1, resuspended in the same buffer and broken by one passage through a French pressure cell at about 3000 lbf in⁻² (20.7 MPa). After centrifugation at 2000 *g* for 5 min to remove unbroken cells (a very small pellet), the cell envelopes were sedimented by centrifugation at 10000 *g* for 15 min at 4 °C. The envelope fraction was washed twice by resuspension in 10 mM-HEPES, 10 mM-MgCl₂ buffer, adjusted to pH 7.4 with NaOH, followed by centrifugation as above. Triton X-100 was added to a concentration of 2% (v/v) and the mixture incubated at room temperature for 20 min. After centrifugation at 10000 *g* for 15 min at 4 °C, the pellet, i.e. the outer membrane fraction, was washed twice in 10 mM-HEPES buffer containing MgCl₂ as above.

Fig. 1. SDS-PAGE profiles of Coomassie brilliant blue stained outer membrane proteins of *E. plautii* VPI 0310 (lane A), VPI 4145 (lane C), *E. saburreum* L32 (lane B) and *F. nucleatum* Fev 1 (lane D). Molecular mass standards are indicated on the left.

Radioiodination of bacteria. Labelling with ^{125}I was done according to the method of Marchalonis (1969). To 200 μl cell suspension (5×10^8 cells ml^{-1}) in 0.02 M-sodium phosphate buffer, pH 7.1, containing 0.9% (w/v) NaCl, was added 500 μCi (18.5 MBq) Na^{125}I , 5 μg lactoperoxidase and 8×10^{-5} M- H_2O_2 . The reaction was stopped after 10 min with 10 μl 1 M-2-mercaptoethanol, followed by 800 μl cell suspension. The cells were sedimented (10000 g for 10 min) and Triton X-100 insoluble cell envelope material was prepared as described above. After analysis by SDS-PAGE, the gels were laid onto Kodak X-Omat films for autoradiography. The films were scanned with a Zeineh scanning densitometer, soft laser 630 nm, adjusting in each case the intensity of the most dense peak to about 80% of full scale.

SDS-PAGE. Membrane proteins were separated by SDS-PAGE according to the method of Laemmli (1970). The concentration of polyacrylamide in the separating gel was 15% (w/v). The proteins were stacked in 4% polyacrylamide gel. Samples were heated in the sample buffer at 100 $^\circ\text{C}$ for 5 min. Samples containing 10–30 μg or 0.1–1 μg protein were applied to gels when they were stained with Coomassie brilliant blue R250 or silver, respectively. Silver staining was done according to Marshall & Latner (1981), with the modifications that all steps were done at 50 $^\circ\text{C}$, using ammonia in the diamine step, and 2% (v/v) glutaraldehyde. Bio-Rad low molecular mass protein standards were used as size markers.

Protein estimation. This was done by a modified Lowry method as outlined by Markwell *et al.* (1978).

Fig. 2. Autoradiography scans of SDS-PAGE gels stained with Coomassie brilliant blue. Molecular masses are indicated at the bottom.

RESULTS

The SDS-PAGE patterns of the outer membrane fractions of *E. plautii* VPI 0310 and VPI 4145 were virtually identical (Fig. 1, lanes A and C). The pattern was distinct and reproducible, and was characterized by a rather large number of low- and high-molecular-mass proteins in large amounts. Similarly, the outer membrane fraction of *E. saburreum* L32 was resolved into a large number of proteins, but the pattern was quite distinct from that of VPI 0310 and VPI 4145 (Fig. 1, lane B). In contrast, the outer membrane protein pattern of *F. nucleatum* Fev 1 showed relatively few bands.

The protein patterns of the envelope fractions of the *Eubacterium* strains were strikingly similar to that of the outer membranes (not shown). In agreement with this the amount of protein in the Triton X-100 soluble fraction was very small. The envelope fraction of *F. nucleatum* Fev 1 had more heavily stained bands than did the outer membrane fraction. A substantial amount of the protein in the envelope fraction was soluble in Triton X-100 (see also Bakken & Jensen, 1986).

Cell surface exposed proteins were compared by iodination of living cells. Following SDS-PAGE and autoradiography scans of the profiles were compared. Fig. 2 shows the relative amounts of radioactivity and molecular masses of the proteins of the outer membrane fractions of VPI 0310 and VPI 4145 compared to Fev 1. Again the profiles of the *E. plautii* strains VPI 0310 and VPI 4145 were apparently identical and distinctly different from that of *F. nucleatum* Fev 1. Proteins soluble in Triton X-100 were not labelled and neither were the cytoplasmic proteins.

Lipopolysaccharides or other carbohydrate materials were looked for in SDS-PAGE gels stained with silver. *F. nucleatum* Fev 1 gave clear positive reactions, mainly in the low-molecular-mass region, but also in the 50–70 kDa region. Weak positive reactions were obtained with the L32 outer membrane preparation, whereas the outer membrane preparations of *E. plautii* VPI 0310 and VPI 4145 gave completely negative reactions.

DISCUSSION

The similarity in outer membrane banding pattern and profiles of surface exposed proteins indicate a near clonal relationship between *E. plautii* VPI 0310 and VPI 4145. In fact, they may be representatives of the same clone. The multitude of Triton X-100 insoluble proteins and the lack of silver stainable material in the outer membrane preparation is compatible with a Gram-positive cell wall structure. In particular, the outer membranes of VPI 0310 and VPI 4145 contained no major protein in the 30–40 kDa range, which is typical of Gram-negative bacteria. The results of this study are thus in agreement with the previous finding of a whole cell fatty acid pattern lacking 3-hydroxy fatty acids (Jantzen & Hofstad, 1981).

The relationship of the proposed species *E. plautii* to other *Eubacterium* species may be questioned. *E. plautii* stains Gram-negative, and the outer membrane protein pattern is quite different from that of *E. saburreum* L32. Diaminopimelic acid (Dpm) was absent in peptidoglycan preparations from *E. plautii* VPI 4145, which contained several amino acids, among them lysine and aspartic acid (Vasstrand *et al.*, 1982). In contrast, *Eubacterium tenue* (Weiss *et al.*, 1981) and *E. saburreum* (O. Kandler, personal communication) have a directly cross-linked m-Dpm type of peptidoglycan. The LL-Dpm type is found in *Eubacterium lentum* and *Eubacterium combesii* (Weiss *et al.*, 1983). The rare subgroup B2 α peptidoglycan is present in *Eubacterium limosum* (Schleifer & Kandler, 1972). Obviously, the genus *Eubacterium* is heterogeneous.

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