Gardnerella vaginalis has a gram-positive cell-wall ultrastructure and lacks classical cell-wall lipopolysaccharide

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Summary. *Gardnerella vaginalis* has a very thin cell wall with a characteristic gram-negative staining pattern and an apparent lamellar structure when viewed at an oblique angle by electronmicroscopy. Examination at right angles to the cell-wall plane and by freeze-etching showed absence of an outer membrane or any other lamellar structure. Cell-wall extracts made by methods specific for lipopolysaccharide (LPS) gave negative reactions by silver staining and for endotoxin in the limulus amoebocyte lysate assay. 2-Keto-3-deoxy-D-manno-2-octonoic acid (KDO), heptose and hydroxy fatty acids specific for LPS were not detected in the extracts. *G. vaginalis* cell walls are unequivocally gram-positive in their ultrastructural characteristics and chemical composition.

Introduction

Gram's stain generally distinguishes two different types of bacterial cell wall, conveniently called gram-positive and gram-negative. These differ radically in chemical composition and molecular architecture (Costerton *et al.*, 1974; Beveridge, 1988) and these structural differences form the basis of significant differences in their sensitivity to antibiotics and in enzyme release patterns (Beveridge, 1988). Gram-negative bacteria are characterised by an elaborate outer membrane that lies outside the cytoplasmic membrane and regulates the passage of solutes into the periplasmic space (Costerton *et al.*, 1974). Paradoxically, Gram's stain clearly identifies gram-positive bacteria that have thick and intact matrix-like cell walls, but it identifies as gram-negative not only those with the typical, double membrane, gram-negative cell-wall structure but also bacteria that have thin, aged, or damaged cell walls (Beveridge, 1988). An example is *Butyrivibrio fibrisolvens* which has a wall of classic gram-positive chemistry and molecular architecture, completely lacking the gram-negative outer membrane, but which is gram-negative because the cell wall is too thin (c. 15 nm) to retain the violet stain (Cheng and Costerton, 1977).

The nature of the cell wall of *Gardnerella vaginalis* has long been a subject of dispute. It does not retain crystal violet during conventional gram-staining procedures, but displays gram-positive features such as definite septum formation during cell division (Reyn *et al.*, 1966). The antibiotic sensitivity pattern of *G. vaginalis* also resembles that of gram-positive bacteria (Piot *et al.*, 1980). Transmission electronmicroscopy (TEM) of sectioned material has shown that the cell walls of *G. vaginalis* are thin and somewhat lamellar, and this structure has been variously interpreted as "typically gram-positive" (Reyn *et al.*, 1966), "trilaminar" (Criswell *et al.*, 1972), and "more closely resembling that of a gram-negative (organism)" (Greenwood and Pickett, 1980).

Analysis of the cell-wall composition of *G. vaginalis* reveals that only 20% of the total cell-wall weight is peptidoglycan (Criswell *et al.*, 1971) which is rather low for a gram-positive organism. Criswell *et al.* (1971) also found many amino acids, including lysine but not diaminopimelate (DAP), present in the peptidoglycan; therefore, they described the organism as a gram-negative bacterium. Their results have been refuted by Piot *et al.* (1980), Harper and Davis (1982) and O'Donnell *et al.* (1984) who, in contrast, found simple amino-acid profiles that included lysine. DAP, common to

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many gram-negative cell walls, and found in a few gram-positive species (Harper and Davis, 1982), was not found. Subsequently, the diamino acid in the *G. vaginalis* wall was confirmed as lysine (O'Donnell *et al.*, 1984), thus explaining the absence of DAP. The studies of Criswell *et al.* (1971, 1972) have been further faulted because the amino-acid profiles for their control organisms were at variance with currently accepted work (O'Donnell *et al.*, 1984; Harper and Davis, 1982).

Ribitol teichoic acid was reportedly absent from the cell walls of *G. vaginalis* (Criswell *et al.*, 1971) but it is not universally present in gram-positive cell walls. Greenwood and Pickett (1980) prepared material from the cell wall of *G. vaginalis* by hot aqueous phenol extraction; they regarded it as a "lipopolysaccharide (LPS)-like" substance. This did not contain 2-keto-3-deoxy-D-manno-2-octonoic acid (KDO) (Greenwood and Pickett, 1980; Piot *et al.*, 1980) or hydroxy fatty acids (Greenwood and Pickett, 1980; O'Donnell *et al.*, 1984) and gave positive results for endotoxin in the limulus amoebocyte lysate (LAL) assay only at very high concentrations (Greenwood and Pickett, 1980). Heptose sugar and dideoxy sugar are also absent from the cell walls of *G. vaginalis* (Harper and Davis, 1982). Therefore, it is doubtful whether *G. vaginalis* contains LPS, at least of the classical variety. The "LPS-like" substance of Greenwood and Pickett (1980) could be a lipoteichoic acid because they share certain characters with lipopolysaccharides (Wicken and Knox, 1975). We present here the results of further ultrastructural and chemical studies of the *G. vaginalis* cell wall.

Materials and methods

Bacterial strains and growth conditions

G. vaginalis ATCC 14018 and clinical isolates 7642, 7803 and 7644 were examined. They were grown in Brain Heart Infusion Broth (Difco Laboratories, Detroit, MI, USA) supplemented with Proteose Peptone Number 3 (Difco) 5% w/v and soluble starch (Difco) 5% w/v, to mid-exponential phase with shaking at 37°C in a CO₂ incubator (Hotpack, Waterloo, Ontario).

Transmission electronmicroscopy, TEM

Bacteria were pre-fixed by the addition of nine parts of glutaraldehyde 5% v/v to one part of broth culture, for 2 h at 22°C. After spontaneous settling of bacteria, the broth + pre-fixative was decanted and the cells were fixed in ruthenium red 0.15% w/v and glutaraldehyde 5% v/v in 100 mM cacodylate buffer, pH 7.2, for 2 h at 22°C. All

subsequent washing used this buffer but with the inclusion of ruthenium red 0.05% w/v. After two washes, the bacteria were agar-enrobed and washed five times. They were then post-fixed in buffer containing OsO₄ 2% v/v for 2 h at 22°C. After five washes, the agar cores were dehydrated in an acetone series (30%, 50%, 70%, 90% and 100%, v/v). The first three dilutions were in buffer but the 90% dilution was in double distilled water due to limitations in ruthenium red solubility. Each stage lasted 20 min except the 90% and 100% washes which lasted 5 min to minimise loss of stain. Cores were further dehydrated for two 10-min periods in propylene oxide before they were embedded in low viscosity resin (Spurr, 1969). Sections were cut to c. 60 nm and stained with uranyl acetate and lead citrate (Reynolds, 1963) before carbon stabilisation. They were examined at an acceleration voltage of 50 kV (Model H-600; Hitachi, Tokyo, Japan).

Freeze-etching and scanning electronmicroscopy, SEM

Cultures were pelleted by centrifugation at 3000 rpm, for 10 min, at 4°C and drops of deposit were affixed to ridged copper disks (3 mm diam; Balzers; Technical Marketing, Vancouver, Canada) in the absence of cryoprotective agents and immersed in liquid Freon 22 maintained around its melting point. Freeze-etching (BA 360 M; Balzers) was performed with cleavage at -100°C and was followed by etching of the surface by sublimation for 5 s. The platinum coating was applied from an angle of approximately 45° and specimens were then examined by SEM (Model S450; Hitachi).

LPS extraction

Lyophilised cells were extracted with hot aqueous phenol (Westphal and Jann, 1965) and by an EDTA-enzymatic method (Darveau and Hancock, 1983). Absorbance scans (A₃₀₀-A₂₀₀) were performed on final extracts to monitor protein and nucleic acid contamination.

Chemical analysis of LPS

Lyophilised LPS prepared by both of the above methods was submitted to a limited range of chemical analyses. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) with 12% gels was used to prepare LPS for subsequent silver periodate staining (Tsai and Frasch, 1982). Assays for (KDO) were as described by Osborn *et al.* (1972) except that a 15-min hydrolysis in 0.5 N sulphuric acid was included (Darveau and Hancock, 1983). Heptose, an LPS-specific sugar, was assayed as described by Wright and Reber (1972) with D-glycero-L-mannoheptose as standard. The presence of 3-hydroxy C₁₄-C₁₈ fatty acids was determined by GLC as described by Kropinski *et al.* (1982). The reactivity of extracts in the LAL test was also determined (Hochstein *et al.*, 1983).

Results

Electronmicroscopy

Cells of all four strains had thin walls (8–12 nm) that were sometimes lamellar but never contained the outer membrane typical of gram-negative cell walls (fig. 1, a–d). *G. vaginalis* walls were fibrillar and unstructured and, where the clear resolution of the underlying cytoplasmic membrane indicated that the sectioning was at right angles to the cell surface (fig. 1, a–d, thick arrows), they were seen to be separated from this inner structure by an electron-transparent zone. In other areas in which the cell envelope was not sectioned at right angles, an illusion, based on Moiré patterns, of lamellar cell-wall structure was produced (fig. 1a and 1c, asterisk) or cell walls appeared thickened (fig. 1b and 1d, asterisk). A small proportion of cells produced thickened septa (fig. 1b, S) and fibrillar residua of dehydration-condensed extracellular polysaccharides (fig. 1a, c and d; fig. 2b, thin arrows) were seen in many of these organisms. Dividing cells of all four strains produced the well-defined septa characteristic of gram-positive cells. A small proportion of cells produced thickened septa when their peripheral cell walls were thin (fig. 1b, S) or thick (fig. 2b, S). Freeze-etching of cells of all four strains showed the absence of an outer cleavage phase and demonstrated thick septa (fig. 2a, S). The thick peripheral walls of cells (fig. 2b) were generally fibrillar and unstructured and sometimes attained a thickness (c. 50 nm) typical of the cell walls of conventional gram-positive organisms. Electron-dense strands of condensed exopolysaccharide material were seen at the surfaces of many of these cells and these condensed strands sometimes appeared to mediate cell-to-cell aggregation (fig. 1a and 1d, thin arrows).

LPS investigations

Absorbance scans indicated that at 280 nm and

260 nm, there was <0.1% of protein and <1.0% of nucleic acid; the Darveau-Hancock preparations were even cleaner (data not shown). Furthermore, proteins were not detected in silver-stained preparations of SDS-PAGE gels. Silver-staining reactions were not obtained with extracts from any of the *G. vaginalis* strains or the negative control ATCC 27217. In contrast, and further confirming correct experimental procedure, a smooth strain of *Salmonella typhimurium* gave a full LPS "ladder" pattern and a rough strain gave a smear at the bottom of the gel, characteristic of rough LPS (data not shown). For convenience, and because no significant differences were detected between LPS extracts prepared by the two methods, results averaged out for each organism are presented in the table. Only the *Salmonella* strains gave reactions typical of gram-negative bacteria, with the expected differences between smooth and rough variants.

Discussion

It is acknowledged that ageing affects the integrity of the cell wall both ultrastructurally and in Gram's stain (Beveridge, 1988) and gram-positive envelope characteristics are known to reflect sampling positions during the growth cycle (Domingue *et al.*, 1988). The results of our electron-microscopy emphasise the value of using actively growing bacteria.

The sections used for examination of the ultrastructure of bacterial cell walls have a finite thickness and, therefore, very thin structures, such as the cytoplasmic membrane (c. 8.5 nm), must be sectioned almost exactly at right angles for correct resolution. Where the cytoplasmic membrane is properly resolved (fig. 1, a–d, thick arrows), the cell wall would be expected to lie at or close to a right angle and the walls of most cells of four strains of *G. vaginalis* were clearly very thin (8–12 nm), homogeneous, fibrillar structures when observed in

Table. Chemical analysis of cell wall extracts of strains of *G. vaginalis*, *Salmonella* and *Staph. aureus*

Strain	KDO ($\mu\text{g}/\text{mg}$)*	Heptose ($\mu\text{g}/\text{mg}$)	C14–18:OH (detection in GLC)	LAL (EU/mg)†
<i>G. vaginalis</i> ATCC 14018	0	0	–	0.17
<i>G. vaginalis</i> VGH 7803	0	0	–	0.02
<i>S. typhimurium</i> (smooth)	0.52	0.05	+	15 900.00
<i>S. typhimurium</i> (rough)	0.17	0.03	+	1190.00
<i>Staph. aureus</i> ATCC 27217	0	0	–	0.01

* Units/mg of LPS extract (dry weight).

† Endotoxin units/mg of LPS extract (dry weight).

Fig. 1. Electronmicrographs of ruthenium red-stained sections of cells of *G. vaginalis*: (a) ATCC strain # 14018; (b) clinical isolate # 7642; (c) clinical isolate # 7803; and (d) clinical isolate # 7644. An electron-transparent zone (thick arrows) is apparent between the cytoplasmic membrane and the very thin wall when the envelope is sectioned at right angles to the cell surface. When sectioning is not at right angles, either a Moiré-based illusion of lamellar walls is produced (asterisk; a and c) or walls may appear thickened (asterisk; b and d). Thickened septa (S) are found in the presence of thin peripheral cell walls (b). Electron-dense, dehydration-condensed, fibrillar exopolysaccharide is present (thin arrows; a, c, d). Bars = 0.1 μ m.

Fig. 2 (a) Electronmicrograph of a freeze-etched preparation of cells of *G. vaginalis* (strain 7642) showing the absence of an outer-membrane cleavage plane and the presence of a thick septum (S) of gram-positive type. (b) Electronmicrograph of a ruthenium red-stained section of cells of *G. vaginalis* (strain 7803) showing the rare presence of both thickened septa (S) and thickened peripheral cell walls. Electron-dense, dehydration-condensed fibrillar exopolysaccharide (thin arrows) are seen at the cell surface. Bars = 0.1 μ m.

these areas. No outer-membrane structures were seen within these cell walls and, in addition, outer-membrane cleavage planes were not demonstrable by freeze-etching, indicating the absence of an outer membrane (Costerton *et al.*, 1974).

Occasional cells of all four strains produced thick septa of the classic gram-positive type and, less commonly, some cells produced thick peripheral cell walls that were similarly homogeneous without signs of an outer-membrane structure. These thick structures resembled those of conventional gram-positive bacteria in thickness (c. 50 nm), in fibrillar homogeneity, and in the complete absence of an outer-membrane structure.

The thinness of the walls of most cells of this organism can account for their tendency to stain gram-negative or gram-variable (Cheng and Costerton, 1977). The lamellar appearance produced when these thin structures are seen at an oblique angle (fig. 1a–d, asterisk) may account for repeated reports of layered cell wall structures in this organism (Criswell *et al.*, 1971, 1972; Greenwood and Pickett, 1980).

To support electronmicrographic data, gram-positive and gram-negative reference strains that included a *G. vaginalis* control and a clinical isolate (7803) were treated with extraction methods for LPS. These methods yielded products that gave expected reactions for all control strains in a limited range of tests. The *G. vaginalis* strains were similar

to *Staphylococcus aureus* in that negative reactions were obtained in tests that gave positive results with the salmonellae. KDO values for the smooth *Salmonella* strain were probably underestimates because of interference in the assay by abequose present in the O antigen. However, reactions with the silver stain, and in GLC for the LPS-specific hydroxy fatty acids, confirmed our methodology and extraction procedures. Also, both *G. vaginalis* strains gave negative reactions in the LAL assay which, though not specific for LPS, is much more sensitive to it than to other amphiphiles such as lipoteichoic acid (LTA) (Wicken and Knox, 1980). It may be that *G. vaginalis* does not contain classical LPS or LTA but rather an entity related to the membrane lipocarbohydrate antigen of Sharp and Poxton (1986).

We conclude, with Reyn *et al.* (1966), that at the ultrastructural level the cell walls of *G. vaginalis* show gram-positive organisation, but that these structures are unusually thin in most cells thereby contributing to the mistaken assumption that they are gram-negative. This conclusion is supported by our chemical analysis which is in accord with more recent reports.

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