Arch Microbiol (1999) 172 : 139–149

ORIGINAL PAPER

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Isolation and characterization of a *Clostridium* sp. with cinnamoyl esterase activity and unusual cell envelope ultrastructure

Received: 11 February 1999 / Accepted: 28 May 1999

Abstract Microorganisms that hydrolyse the ester linkages between phenolic acids and polysaccharides in plant cell walls are potential sources of enzymes for the degradation of lignocellulosic waste. An anaerobic, mesophilic, spore-forming, xylanolytic bacterium with high hydroxy cinnamic acid esterase activity was isolated from the gut of the grass-eating termite *Tumilitermes pastinator*. The bacterium was motile and rod-shaped, stained gram-positive, had an eight-layered cell envelope, and formed endospores. Phylogenetic analysis based on 16S rRNA indicated that the bacterium is closely related to *Clostridium xylanolyticum* and is grouped with polysaccharolytic strains of clostridia. A wide range of carbohydrates were fermented, and growth was stimulated by either xylan or cellobiose as substrates. The bacterium hydrolysed and then hydrogenated the hydroxy cinnamic acids (ferulic and *p*-coumaric acids), which are esterified to arabinoxylan in plant cell walls. Three cytoplasmic enzymes with hydroxy cinnamic acid esterase activity were identified using non-denaturing gel electrophoresis. This bacterium possesses an unusual multilayered cell envelope in which both leaflets of the cytoplasmic membrane, the peptidoglycan layer and the S layer are clearly discernible. The fate of all these components was easily followed throughout the endospore formation process. The peptidoglycan component persisted during the entire morphogenesis. It was seen to enter the septum and to pass with the engulfing membranes to surround the prespore. It eventually expanded to form the cortex, verification for the peptidoglycan origin of the cortex. Sporogenic vesicles, which are derived from the cell wall peptidoglycan, were associated with the engulfment process. Spore coat fragments appeared early, in stage II, though spore coat formation was not complete until after cortex formation.

Key words *Clostridium xylanolyticum* · Cinnamic acid · Esterase · Lignocellulose · Sporogenesis · Ultrastructure · Cell envelope

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Introduction

Hydroxycinnamic acids, of which ferulic and *p*-coumaric acids are the main constituents, represent 1–5% (w/w) of dry-season tropical grasses (Lowry et al. 1993). These two phenolic acids are ester-linked to arabinoxylan and lignin, or cross-linked to lignin and polysaccharide through ether and ester bonds, respectively. It is thought that the ester linkages between phenolic acids and polysaccharide limit the anaerobic degradation of fibre by microorganisms in the gut of ruminants (Bacic et al. 1988; Jung and Deetz 1993). Enzymes that hydrolyse these ester linkages are a potentially important resource for the degradation of lignocellulose-containing waste. Consequently, a range of anaerobic environments were screened for bacteria that demonstrated high activities for hydroxy cinnamic acid esterase. In this process, a facultative anaerobic bacterium, *Clostridium* sp. strain LP1, was isolated from the grass-eating termite *Tumilitermes pastinator*. This paper reports on the isolation, phylogenetic analysis, physiological properties, and unusual cell-envelope ultrastructure of this bacterium. It also describes the involvement of these envelope layers in the formation of endospores.

Materials and methods

Termites

Grass-eating termites of the species *T. pastinator* were collected from earthen mounds in their natural environment near Townsville

in tropical North Queensland, Australia. The termites were separated from contaminating debris, held in a glass flask at ambient temperature, and used within 24 h of collection. They were taken into an anaerobic chamber with a 95% CO_2/5% H_2 gas phase, suspended in anaerobic salt diluent (1 g/5 ml), and processed for 5 min with a homogenizer (Sorvall Omni-Mixer; Watson and Victor, Australia). One millilitre of the homogenate was inoculated into 10 ml of anaerobic enrichment medium.

Anaerobic techniques, media, and isolation procedure

The anaerobic techniques of Hungate (1969) as modified by Bryant (1972) were used for the growth of organisms and the preparation of media. Basal medium contained 30% (v/v) clarified rumen fluid, 3.75% (v/v) minerals 1 and 2 (Caldwell and Bryant 1966), 1 mM sodium formate, 0.1% (v/v) Pfennig's trace minerals, 0.4% (w/v) Na_2CO_3, 0.05% (w/v) cysteine hydrochloride and 0.001% (w/v) resazurin. The media were gassed with CO_2, dispensed into 25-ml Balch tubes (18 × 250 mm), stoppered, and autoclaved for 15 min at 15 psi. B-vitamins (Lowe et al. 1985) were added to each tube of medium just prior to inoculation, and incubations were at 39 °C in the dark. Ferulic acid (1 mM) and *p*-coumaric acid (1 mM) were added to basal media to enrich for organisms with phenolic acid esterase that may utilize the hydrolysed acids as carbon sources for growth. Butler and Buckerfield (1979) have inferred that microorganisms in the gut of termites can degrade phenolic acids from the cell walls of plants. The initial culture in this study was serially transferred through enrichment media at 2-day intervals for 8 days. The final enrichment culture was inoculated onto agar enrichment media after dilution to 10^{-3} and 10^{-6}. Cultures were considered to be pure after three successive isolations from agar plates showed a single colony morphological type.

Morphology

Living cells and cells stained by the Gram reaction were examined by light microscopy. For electron microscopy, cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4 °C without exposing them to air. These were subsequently encased in 2% agarose (low-gelling-temperature agar; Sigma Type VII) and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature. After dehydration through a graded ethanol series, the specimens were infiltrated with epon resin and polymerized at 60 °C for 24 h. Some cells were treated with 150 µg lysozyme (Sigma) per millilitre of 50 mM Tris for 2 h at 22 °C before being fixed and processed as above. Ultra-thin sections were cut using a Reichert-June Ultracut E microtome and were stained with 5% uranyl acetate in 50% methanol and Reynolds' lead citrate. Whole cells were negatively stained with 1% uranyl acetate or shadowed with platinum-carbon using a Balzers BAE 120 vacuum apparatus. All preparations were viewed in a Hitachi H-800 or a JEOL 1010 transmission electron microscope.

Growth and nutrition studies

Utilization of substrates and production of end products of fermentation were tested in basal medium containing 0.05% each of Casitone (Difco, Detroit, Mich., USA), glucose, cellobiose, birchwood xylan (Sigma), starch (soluble) and maltose, 0.02% Na lactate, and 1 mM ferulic and *p*-coumaric acids (TCC medium) or containing various combinations of these substrates. Growth in basal medium containing 0.05% Casitone and 0.5% spear grass (*Heteropogon contortus*) was also determined. The effect of H_2 on the growth rate was determined in cultures with an H_2/CO_2 (1:1) gas phase and basal medium. The Microscan (Baxter Healthcare, West Sacramento, Calif., USA) and the Microbact System 24 AN (Disposable Products, Ridleyton, South Australia) for identification of anaerobic bacteria by biochemical substrate reactions were also used.

Tests for antibiotic sensitivity were based on inhibition of growth in TCC broth medium with tetracycline (20 µg/ml) and chloramphenicol (25 g/ml) and with Microring antibiotic discs on TCC agar medium (Medical Wire and Equipment, Victory Gardens, N.J., USA) containing erythromycin (60 mg), rifampicin (15 mg), colistin (10 mg), penicillin G (2 units), kanamycin (1,000 mg), and vancomycin (5 mg).

Analytical methods

Volatile fatty acids, lactate, succinate, phenylacetate, and pyruvate were analysed on a Varian 3700 gas chromatograph [flame ionization detection (FID)] using a DB-Wax Megabore column (30 m × 0.53 mm; J & W Scientific) with a 1-mm film thickness at temperature-programmed conditions according to the methods of Holdemann et al. (1977). A gas-liquid chromatograph (Shimadzu 9A) was used to determine ethanol (glass Carbowax 1500 column, 2 m × 0.2 mm at 90 °C with helium carrier gas, FID detection), methane and H_2 (Porapak Q 100/120 column at 35 °C with nitrogen carrier gas, FID and thermal conductivity detection) concentrations in cell cultures.

The G+C content of cellular DNA was determined by the thermal melt method as described by Sly et al. (1986) with DNA from *Escherichia coli* used as a reference. Ester-linked ferulic acid and *p*-coumaric acid were extracted from plant samples and culture residues as described previously (McSweeney et al. 1994). Metabolism of phenolic monomers was monitored by the HPLC procedure of Jung et al. 1993. Thirty microlitres of culture fluid was injected into a reverse-phase analytical column (8 × 100 mm; Waters Nova-Pak C18, Radial-pak cartridge, 4-mm particle size) with a mobile phase of water/glacial acetic acid/butanol (350:1:7) that was eluted isocratically at 2 ml/min. Phenolic metabolites were collected as individual fractions as they eluted from the analytical column, were lyophilized, and were analysed by mass spectrometry under electron impact with a Kratos MS-25 RFA magnetic sector instrument. Mass spectrometer conditions were: ion source temperature, 200 °C; scan rate, 1 s/decade; and ionizing voltage, 70 eV. Each compound was considered to be positively identified when its mass spectrum was the same as that of an authentic standard.

Amplification and sequencing of the 16S rRNA gene

Bacterial biomass of strain LP1 cultured in TCC media for 2 days was centrifuged, washed once in sterile distilled water, and frozen at –20 °C. Methods detailed in Blackall (1994) were employed for isolation and storage of DNA and polymerase chain reaction (PCR) amplification of the 16S rRNA gene. A mixture of manual sequencing (Dorsch and Stackebrandt 1992) and automated sequencing (Blackall 1994) was employed to obtain the near-complete sequence of the 16S rRNA gene from strain LP1. Primers for PCR (primers 27f and 1492r) were employed; for sequencing, primers 343r, 519r, 787r, 907r, 1100r, 1241r, 1385r and 1492r were used. The GenBank accession number for the nucleotide sequence of *C. xylanolyticum* LP1 is AF146699.

Comparative sequence analysis

Sequence data were compiled in the editing program SeqEd (Applied Biosystems, Foster City, CA, Calif., USA). They were manually aligned against reference *Clostridium* sequences (Rainey and Stackebrandt 1993) obtained from the RDP using the AE2 editor (Larsen et al. 1993). The alignment was verified using secondary structural constraints of the 16S rRNA molecule. Due to the high relatedness of the strains used in the analysis, variable regions of the sequences had identical secondary structure and were included in the analysis. Trees were constructed using distance methods in PHYLIP (phylogeny inference package) version 3.5 (Felsenstein 1993: http://evolution.genetics.washington.edu/phylip.html) and maximum parsimony methods available in PAUP (phylogenetic

analysis using parsimony) version 3.1.1 (Swofford 1993: Illinois Natural History Survey, Champaign, Ill.). An evolutionary distance matrix was prepared using the Jukes and Cantor-parameter model in DNADIST and trees constructed using the neighbour-joining method with Neighbor. To quantify relative support for branches inferred from genetic distance analyses and parsimony, bootstrap resampling was employed. Ninety-five percent was taken to be significant for hypotheses proposed a priori after Felsenstein (1985). *Clostridium cellulovorans* was used for out-group comparison. This bacterium is closely related to *C. xylanolyticum*, but is distinct from it (Rainey and Stackebrandt 1993).

Preparation of cultures for enzyme assays

Cells growing exponentially in TCC media were separated from culture fluid by centrifugation (7,000 × *g* for 20 min at 4 °C). Cell pellets were washed and suspended in 0.1 M Bis-tris [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; pH 7] and centrifuged as described. The cell pellet was resuspended in 0.01 × the original volume and disrupted twice by ultrasonication with a Sanophon ultrasonic disintegrator (Ultrasonic Industries, Sydney, Australia) at 60 W for 10 min at a time. The cell preparation was then centrifuged (65,000 × *g* for 40 min) with a Beckman TL-100 ultracentrifuge rotor. The supernatant fluid from this procedure was removed and designated the cytosol fraction, while the pellet (cell membrane fraction) was resuspended to the same volume in 0.1 M Bis-tris.

Esterase activity

Feruloyl and *p*-coumaroyl esterase activity was assayed by estimation of the free acids cleaved from [5-*O*-(*trans*)-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) and [5-*O*-(*trans*)-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX), respectively, as described by Borneman et al. (1990). FAXX and PAXX were isolated from spear grass by liquid chromatography after treatment with polysaccharide hydrolases (Borneman et al. 1990). Aryl esterase activity in cellular fractions and extracellular fluid were measured with phenyl acetate and *p*-nitrophenyl acetate (Johnson et al. 1988), α-naphthyl acetate (Hespell and O'Bryan-Shah 1988), α-naphthyl butyrate, α-naphthyl caprylate, α-naphthyl laurate, and 4-methylumbelliferyl acetate (McDermid et al. 1990) as substrates (1 mM). A unit of enzyme is defined as 1 mmol of product formed per minute. All assays were conducted at 39 °C in duplicate with appropriate controls. The protein concentration of bacterial cell fractions was determined by the method of Bradford (1976), with bovine plasma gamma globulin as a standard.

Native polyacrylamide gel electrophoresis assays

Nondenaturing polyacrylamide gels (1.5 mm) were run at 4 °C with a 12% polyacrylamide resolving gel and a stacking gel (pH 7) as described by Ornstein (1964). The resolving gel contained 0.1 M Tris-HCl (pH 8), 12% acrylamide, 0.32% bis-acrylamide, 0.05% ammonium persulfate, and 0.1% N,N,N′,N′-tetramethylethylenediamine. The stacking gel contained 4% acrylamide, 0.13% bis-acrylamide, 86 mM Tris (pH 7), and the remaining components as in the resolving gel. The electrode buffer (pH 8.3) contained 50 mM Tris and 0. 38 M glycine. The gels were stained for esterase activity with α- and β-naphthyl acetate as substrates using the method of Rosenberg et al. (1975). Esterase activity in resolving gels was also detected by fluorescence in a zymogram that contained 4-methylumbelliferyl *p*-trimethyl ammonium cinnamate chloride (MUTMAC; Sigma) as the substrate (Dalrymple et al. 1996). The resolving gel was washed with 0.1 M phosphate buffer at 37 °C and was overlaid with molten agarose solution [0.7% agarose in 0.05 M phosphate buffer (pH 7.4)] containing 0.5 mg MUTMAC/ml. The zymogram was viewed under UV light after reacting for 10 min.

Replicates of the protein samples (1 mg) were run on all activity gels so that some lanes could be stained for esterase activity, while sections in the unstained lane that corresponded with bands of esterase activity were excised and incubated with FAXX and PAXX (0.2 mg/ml) in 300 ml 0.1 M Bis-tris (pH 7).

Results

Isolation, morphology, and cell envelope ultrastructure

Two distinct morphological types were isolated, one being *Clostridium* sp. LP1, which formed dark-cream colonies with irregular edges. This isolate was motile and rod-shaped, produced terminal spores (Fig. 1A), and stained positive to the Gram reaction. Ultrastructural studies showed that the cell envelope of *C. xylanolyticum* consists of eight layers – four dense layers separated by electron-lucent regions (Fig. 1B). In this study, the term "envelope" is used to describe all the cell surface layers that surround the cell cytoplasm, while "wall" refers to the peptidoglycan-based outer region of the envelope lying outside the cytoplasmic membrane (Hancock 1991). Identification of these envelope layers is based initially on comparison with studies on other gram-variable clostridia (Sleytr and Glauert 1976; Beveridge 1990). The inner two dense layers represent the inner and outer faces of the cytoplasmic membrane, the inner one being closely apposed to the cytoplasm. There is an asymmetry between these membrane leaflets, the outer one being 7 nm in thickness while the inner one is only 2–3 nm thick. The outer dense layer of the wall is most likely an S layer. However, none of the repeating proteinaceous array that is usually present in all S layers is visible in thin-section or in negatively stained or metal-shadowed preparations of whole cells. A smooth S layer has previously been seen in a gram-variable bacterium (Beveridge 1990). The finer inner dense layer of the wall is the peptidoglycan-containing matrix. This layer was generally only 2–3 nm thick, though a small number of cross-sections were seen in which it was up to 5 nm thick. The S layer and the outer cytoplasmic membrane leaflet are of similar electron density and width (7 nm). The coat of diffuse material is variable in thickness and often absent, and it possibly represents an extracellular mucopolysaccharide capsule.

Lysozyme treatment of the cells resulted in most cases in the removal of all envelope layers outside the cytoplasmic membrane (Fig. 1C). The S layer may be lost as a result of the breakdown of the peptidoglycan layer. Several cells were observed in which the S layer was still intact around the cell although the peptidoglycan layer was not present (Fig. 1D). Many free S layers were also seen quite separate from any cells in this preparation. These were removed from the cells with the dissolution of the peptidoglycan layer by the lysozyme. Therefore, the initial identification of the envelope layers was verified by the lysozyme experiment.

At the poles of the cell, the outer leaflet of the cytoplasmic membrane (or material closely associated with it)

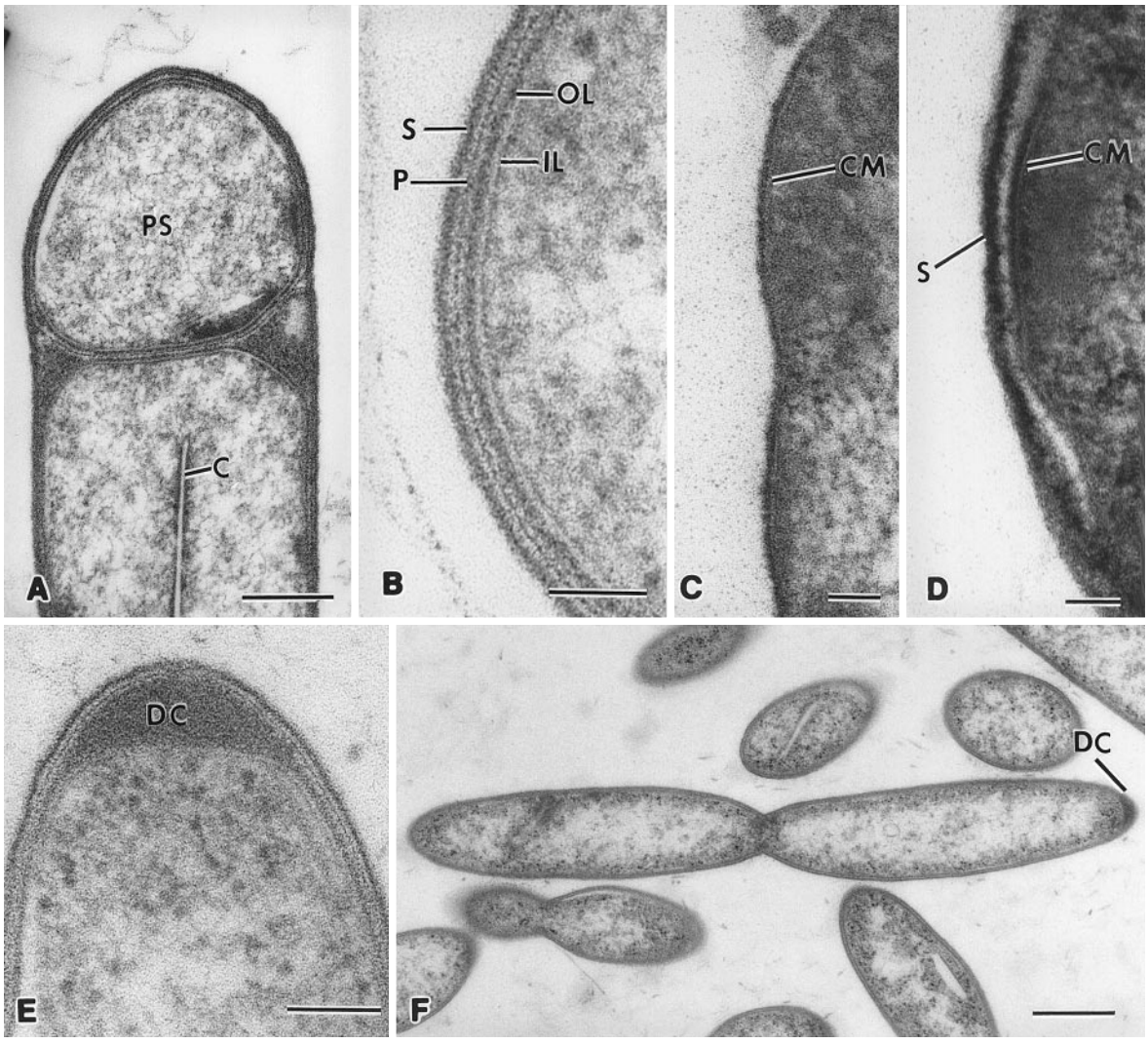


Fig. 1 **A** Longitudinal section of a cell undergoing sporogenesis. A sporulation septum separates the developing prespore (*PS*) from the mother cell. A channel (*C*) is present in the cytoplasm of the mother cell (*bar* 0.2 μm). **B** Cell envelope of *Clostridium xylanolyticum* showing the inner (*IL*) and outer (*OL*) leaflets of the cytoplasmic membrane, and the wall layers (*P* the peptidoglycan layer and *S* the S layer). The coat is absent from this cell (*bar* 50 nm). **C** Cell envelope of a cell of *C. xylanolyticum* after treatment with lysozyme. Only the double track of the lipid bilayer of the cytoplasmic membrane (*CM*) remains on the outside of the cell (*bar* 50 nm). **D** Cell envelope of a cell of *C. xylanolyticum* after treatment with lysozyme. On this cell the cytoplasmic membrane (*CM*) and the S layer (*S*) remain, but the peptidoglycan has been removed (*bar* 50 nm). **E** Pole of a cell showing the cap-like expansion (*DC*) of the outer leaflet of the cytoplasmic membrane (*bar* 0.1 μm). **F** Longitudinal section of a dividing cell possessing a domed cap (*DC*) (*bar* 0.5 μm)

expands, giving the appearance of a domed cap (60–75 nm in thickness) formed from the cytoplasmic membrane leaflet (Fig. 1E, F). The wall layers remain of constant thickness in this region, as does the inner leaflet of the cytoplasmic membrane. During vegetative division, the cell forms a lightly tapered constriction involving all layers of the cell envelope (Fig. 1F). This constriction of the envelope continues until the cell is pinched in two. No septum is formed during this division, although a true septum is formed as the initial stage of sporogenesis (Fig. 1F). Studies of large numbers of thin-sections and of negatively stained and shadowed whole cells showed that this is the only form of vegetative division that occurs and that septa are only formed as the initial stage of sporogenesis (Fig. 1A).

Channels of a constant 10-mm width running longitudinally along the cell for up to 1 mm were seen within the cytoplasm (Fig. 1A). In transverse section, these channels also appear as 10-nm profiles across most of the width of the cell.

Sporogenesis

Sporogenesis in *C. xylanolyticum* can be described in terms of the six distinct morphological stages. Stage II is characterized by the formation of the sporulation septum. During septum formation, the cytoplasmic membrane invaginates and grows until a continuous septum is formed across the cell, separating the prespore and the mother cell

(Fig. 1A). Concurrent with this, the peptidoglycan wall layer divides so that a derivative of it passes into the septum with the invaginating cytoplasmic membrane. This becomes a single central layer in the septum, while the main peptidoglycan layer remains adjacent to the S layer as a continuous wall around the periphery of both the prespore and the mother cell. The septum consists of five dense layers, i.e. two membranes separated by wall material (Fig. 2A, B). Sporogenic vesicles consisting of an obvious double-layered wall develop within a region of dense material derived from the peptidoglycan layer of the wall at the periphery of the septum. Since the peptidoglycan layer can be seen bounding this region on all sides, these vesicles originate solely from the peptidoglycan material. These sporogenic vesicles appear superficially like mesosomes that have been seen associated with the septum in other sporulating bacterial species. However, while mesosomes are derived from the cytoplasmic membrane, these sporogenic vesicles are formed within the peptidoglycan layer of the wall. They do not develop at any other location associated with the spore septum.

The septum appears quite rigid throughout the engulfment process, never becoming irregular as seen in other bacterial species. The cytoplasmic membranes and the peptidoglycan layer remain clearly visible during the entire process.

The junction of the septum and the cell envelope moves towards the spore pole along the sides of the prespore, taking with it the sporogenic vesicles enclosed within the membranes (Fig. 2E). With the fusion of the engulfing membranes from all sides at the pole, the forespore is formed completely enclosed by its own spore envelope (Fig. 2F). The sporogenic vesicles disappear prior to the completion of engulfment, and their fate is unknown.

Short, electron-dense, membranous spore coat fragments develop in the mother cell adjacent to the periphery of the septum (Fig. 2D). Either the spore coat fragments move up around the sides of the prespore with the septum front, or more are synthesized in this region. These join together to form, after engulfment, the spore coat, which is a discontinuous multilayered structure around the forespore (Fig. 2E). The fragments finally coalesce to form a continuous coat after cortex formation has commenced (Fig. 2F).

The initial process of cortex formation (stage IV) is seen after engulfment, when the peptidoglycan wall material between the membranes becomes more electron-dense and expands at certain points (Fig. 2F). The cortex development subsequently becomes even around the entire periphery of the forespore. During its growth, dense inner and outer bands (which are closely associated with the inner and outer membranes, respectively) can be resolved (Fig. 2G, H). Expansions of the inner dense band protrude, with the inner membrane, into the core (Fig. 2H). Associated with the outer membrane is a single layer of granules (Fig. 2H).

The released mature spore has several distinct layers (Fig. 2I, J): core, inner membrane, cortex, outer membrane, residual mother cell cytoplasm and the spore coat. Upon release, the spore coat becomes markedly irregular in shape, though no external architecture or appendages are present on the mature spore.

Growth and antibiotic resistance

Growth occurred both under strict anoxic conditions and when resazurin, the oxidation indicator in culture media, was initially oxidized. Growth did not occur when the organism was continuously exposed to oxygen. Optical densities of growing cultures were greater than 1.0 when either xylan or cellobiose were used for growth. The organism grew on spear grass as a substrate but did not attach to this plant material when viewed by light microscopy. Fermentation tests indicated that a wide range of carbohydrates were utilized, including arabinose, cellobiose, fructose, lactose, maltose, mannitol, mannose, meliobiose, raffinose, rhamnose, ribose, salicin, sucrose, trehalose and xylose. Sorbitol was not utilized. Lactate was utilized but did not support growth as the sole energy source. Growth was not stimulated by the presence of H_2 in an energy-limited medium.

The isolate was sensitive to erythromycin, rifampicin, penicillin, kanamycin, vancomycin, tetracycline and chloramphenicol, but was resistant to colistin.

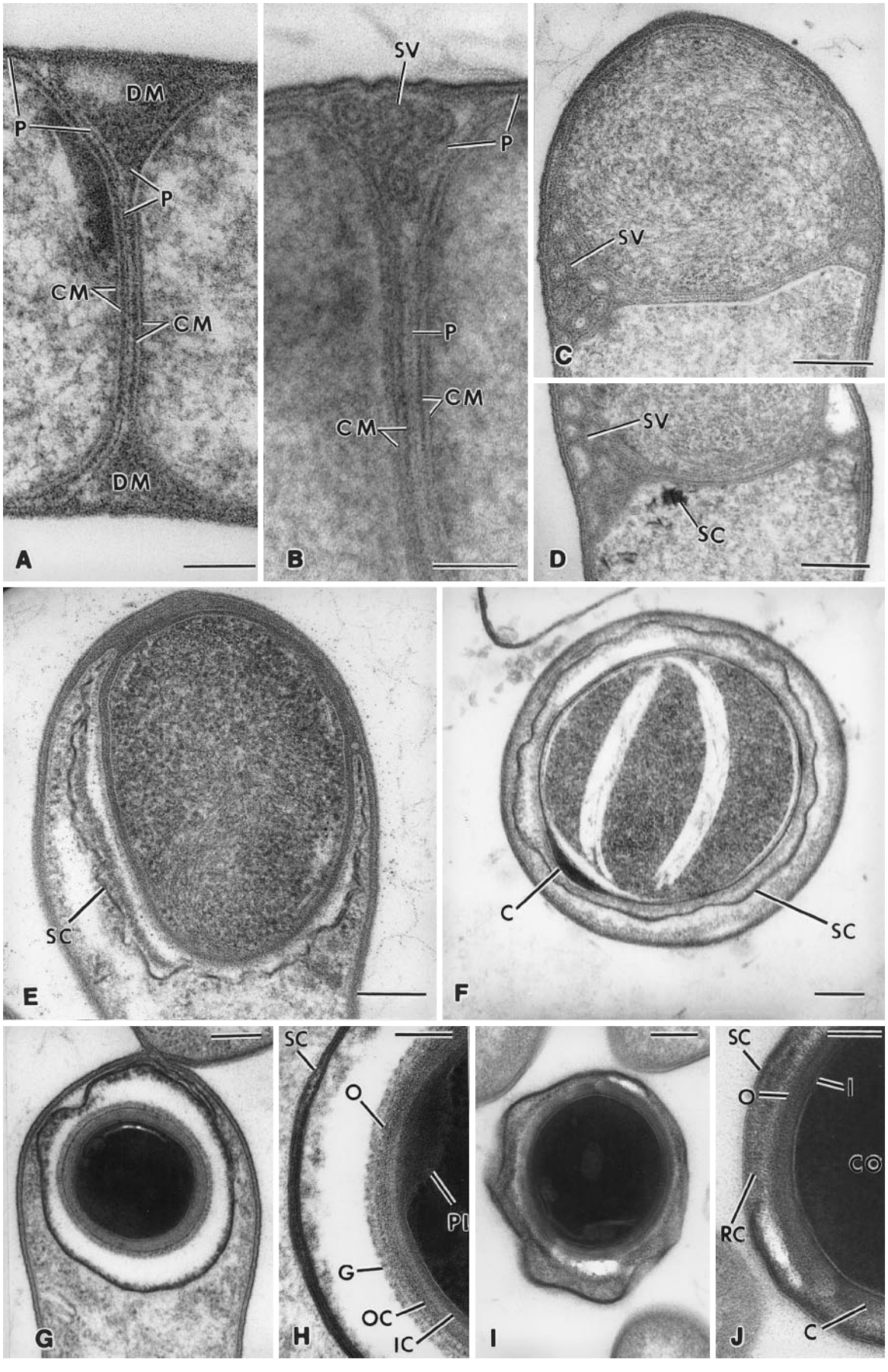
Metabolism

Indole was not produced, nitrate was not reduced, esculin and starch were hydrolysed, but catalase was not detected. Fermentation end products from TCC media were ethanol, acetate, H_2 and CO_2. Pyruvate, succinate, phenylacetate and methane were not produced from this media as end products. Using the Microscan tests, positive results were obtained for α- and β-galactosidase, phosphatase, and trehalase; a strong positive result was obtained for α-glucosidase, and a weak one for β-glucosidase. Negative results were obtained for *N*-acetyl-β-glucosaminidase, phosphodiesterase, α-fucosidase, α-mannosidase, leucine aminopeptidase, methionine aminopeptidase, acid and alkaline lysine aminopeptidase, glycine aminopeptidase, proline aminopeptidase, and arginine aminopeptidase.

Mass spectrometry confirmed that ferulic and *p*-coumaric acids (1 mM) were rapidly (< 24 h) hydrogenated to dihydroferulic and dihydro *p*-coumaric acids, respectively, without further biotransformation or the formation of other intermediates.

Esterase activity

Spear grass contained ester-linked ferulic acid (0.025 mmol/g) and *p*-coumaric acid (0.098 mmol/g), of which 6% and 0%, respectively, were apparently released during a 48-h incubation with the organism. Hydroxy cin-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◀ **Fig. 2** **A** Sporogenesis septum consisting of the two leaflets of each of the two invaginated cytoplasmic membranes (*CM*) and the peptidoglycan (*P*) layer. The peptidoglycan layer of the septum is continuous with that of the wall. A dense material (*DM*) of peptidoglycan origin is laid down in the periphery of the septum (*bar* 0.1 μm). **B** Five layers of the septum: inner and outer leaflets of the two cytoplasmic membranes (*CM*) and a peptidoglycan layer (*P*). Sporogenic vesicles (*SV*) (which were formed at the periphery of the septum, derived solely from peptidoglycan material) (*bar* 0.1 μm). **C** Sporogenic vesicles (*SV*) moving up around the outside of the prespore at the commencement of engulfment (*bar* 0.2 μm). **D** Longitudinal section of a septum at a stage slightly later than that shown in **C** Sporogenic vesicles (*SV*) are elongated as they move up the sides of the prespore. Presumptive fragments of the spore coat (*SC*) have aggregated in the mother cell adjacent to the periphery of the septum (*bar* 0.2 μm). **E** Prespore prior to being fully engulfed. Spore coat fragments (*SC*) form a multilayered discontinuous sheet (*bar* 0.2 μm). **F** Transverse section of a forespore. The initial stage of cortex formation is represented by expansion of the peptidoglycan layer (*C*). A continuous spore coat (*SC*) surrounds the forespore (*bar* 0.2 μm). **G** Well-developed cortex surrounding a forespore and exhibiting the separation of the residual cytoplasm from the outer membrane (*bar* 0.2 μm). **H** High-magnification micrograph of a forespore at the same stage of development as that shown in **G** Dense inner (*IC*) and outer (*OC*) bands are visible in the cortex. An expansion of the inner band protrudes into the forespore cytoplasm (*PI*). A layer of granules(*G*) lies adjacent to the outer membrane (*O*) (*bar* 0.1 μm). **I** Mature spore freed from the mother cell (*bar* 0.2 μm). **J** High-magnification micrograph of a mature spore showing spore coat (*SC*), residual mother cell cytoplasm (*RC*), outer membrane (*O*), cortex (*C*), inner membrane (*I*) and core (*CO*) (*bar* 0.1 μm)

Table 1 Esterase activities of the cell cytosol fraction of *Clostridium xylanolyticum* (*U* mmol of product formed/min)

Substrate	Specific activity (U/mg protein)
Ferulic acid ester (FAXX)	0.055
p-Coumaric acid ester (PAXX)	0.044
Phenyl acetate	0.126
p-Nitrophenyl acetate	0.133
a-Naphthyl acetate	0.138
a-Naphthyl butyrate	0
a-Naphthyl caprylate	0
a-Naphthyl laurate	0
a–4-Methylumbelliferyl acetate	0.001

namic acid esterase activity was associated mainly with the cell cytosol fraction, with negligible amounts in the culture supernatant and cell membrane fraction. Esterase from the cytosol cleaved FAXX, PAXX, phenyl acetate, *p*-nitrophenyl acetate, α-naphthyl acetate and 4-methyl umbelliferone, but exhibited no activity on α-naphthyl butyrate, α-naphthyl caprylate and α-naphthyl laurate (Table 1).

The staining of gels for esterase activity with either α-naphthyl acetate, β-naphthyl acetate, or MUTMAC as substrates revealed the same three distinct bands, although the staining was more intense with β-naphthyl acetate as compared to that with α-naphthyl acetate (Fig. 3). Band 2 had the highest activity against FAXX and PAXX, while bands 1 and 3 had moderate and weak activities, respectively, against both substrates.

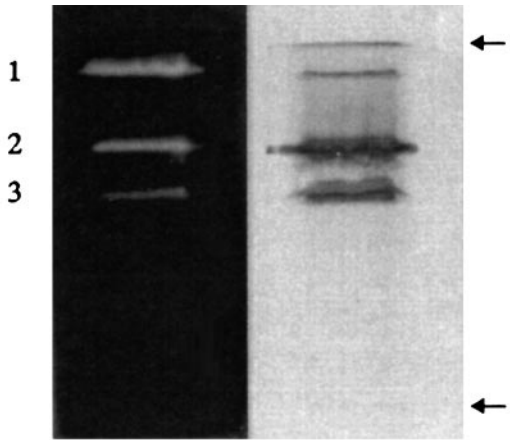

Fig. 3 Zymograms of non-denaturing polyacrylamide gel stained for esterase activity with MUTMAC (*left panel*) and β-napthyl acetate (*right panel*). *Upper* and *lower arrows* indicate the top of the resolving gel and the dye front of the chromatogram, respectively

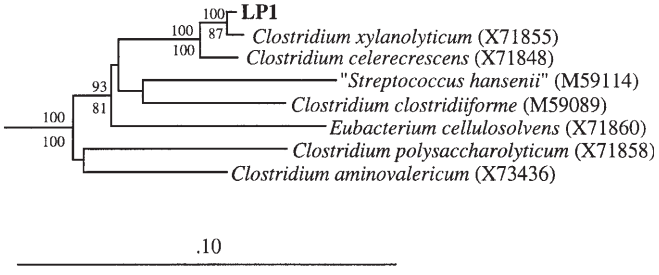

Fig. 4 Evolutionary distance tree of *Clostridium xylanolyticum* strain LP1 and other members of the genus *Clostridium, Eubacterium* and *Streptococcus* based on the comparative analysis of 1,272 nucleotide positions. Bootstrap values greater than 74% (100 bootstrap resamplings) from distance (*upper*) and parsimony (*lower*) analyses are presented at nodes. The outgroup used in the analyses was *Clostridium cellulovorans*

Nucleic acids and 16S rRNA comparisons

The G+C of DNA was 37.8 mol%. A nearly complete 16S rRNA gene sequence for *C. xylanolyticum* strain LP1 (1,383 nucleotides) was obtained. It was most closely related by sequence similarity to *C. xylanolyticum* (strain ATCC 4963; 99.1%) and to *Clostridium celerecrescens* (strain DSM 5628; 97.8%) (Fig. 4).

Discussion

From the phylogenetic analysis and the phenotypic and ultrastructural determinations, strain LP1 was identified as *C. xylanolyticum*. Phylogenetic analysis showed that strain LP1 groups with cluster III of Rainey and Stackebrandt (1993), which includes the polysaccharolytic strains of *Clostridium*. However, strain LP1 differed in substrate range in that *C. xylanolyticum* has not been shown to ferment arabinose, ribose and trehalose (Rogers and Baecker 1991). *C. xylanolyticum* is also closely related (97.8%) to the anaerobic, cellulolytic bacterium *C.*

celerecrescens (Palop et al. 1989; Rogers and Baecker 1991).

The original isolate of *C. xylanolyticum* was grown from decaying pine wood (Rogers and Baecker 1991), while strain LP1 was isolated from a sample of termites and *C. celerecrescens* was enriched from a methanogenic culture of cow manure. The sources of these closely related polysaccharolytic bacteria are quite distinct, although a methanogenic environment may be common to all.

Feruloyl and *p*-coumaroyl esterase activities have been described in several genera of fungi and in the bacteria *Streptomyces* sp., *Fibrobacter succinogenes, Butyrivibrio fibrisolvens* and *Ruminococcus* sp (Akin et al. 1993; Christov and Prior 1993; McSweeney et al. 1998), but this is the first report of these enzymes in a clostridial organism. The specific activities of the clostridial enzymes against FAXX and PAXX were similar (0.01–0.1 U/mg protein) in comparison with data from *F. succinogenes, B. fibrisolvens* and *Ruminococcus* sp. (McDermid et al. 1990; Akin et al. 1993; McSweeney et al. 1998). Acetyl esterase activity was also detected using phenyl acetate, *p*-nitrophenyl acetate and α-naphthyl acetate as substrates. The zymogram showed that some of these acetyl esterases also possessed feruloyl and *p*-coumaryl esterase activities. The intracellular location of feruloyl and *p*-coumaryl esterase activity in strain LP1, and the inability of this organism to release esterified cell wall phenolic acids from insoluble plant substrate indicate that these enzymes are not primarily involved in fibre degradation but probably act on the depolymerized hemicellulose that is assimilated by the organism. Modification of ferulic and *p*-coumaric acids by reduction of the C_3 side-chain double bonds was demonstrated for strain LP1 and has also been observed in another termite isolate, *Clostridium sporogenes* strain KX1 (Kuhnigk et al. 1994).

The previous description of *C. xylanolyticum* (Rogers and Baecker 1991) provided a small amount of information about the ultrastructure of this organism. The cell envelope was described as being five-layered. In the present study it was found to consist of eight layers: four electron-dense layers with an electron-lucent region between each, and an external coat. The inner two dense layers are interpreted as constituting the cytoplasmic membrane, while the outer two (the peptidoglycan-based layer and an S layer) together make up the wall. Such an envelope structure is not common for a gram-positive bacterium. Members of the genus *Clostridium* are generally considered to be gram-positive. However, some species of this group tend to lose their gram-positive reaction early in culture, especially broth culture, and may appear gram-negative (Cato et al. 1986). Rogers and Baecker (1991) have described *C. xylanolyticum* as gram-negative, but they did not indicate whether this observation was based on ultrastructure or Gram stain. Strain LP1 always stained gram-positive in the present study. The ultrastructure of the cell wall of several species of *Clostridium* that display variability to the Gram stain have been studied (Sleytr et al. 1969; Sleytr and Glauert 1976; Beveridge 1990). These species also possess an unusual multilayered cell envelope configuration. Experiments by Sleytr and Glauert (1976) using lysozyme have shown that the inner dense layer of the wall contains peptidoglycan. Rapid exponential growth of these cells produces rapid cell wall turnover that thins the peptidoglycan layer and promotes gram-negativity (Beveridge 1990). Lysozyme treatment of *C. xylanolyticum* in this study also identified the dense layer peripheral to the cytoplasmic membrane of the cell envelope as peptidoglycan.

While the layout of the cell envelope of *C. xylanolyticum* resembles that described for other gram-variable clostridia with a multilayered cell envelope (*Clostridium thermohydrosulfuricum, Clostridium thermsaccharolyticum* and *Clostridium tetani*) (Sleytr and Glauert 1976; Beveridge 1990), the structure of the layers is not identical. The asymmetry of the cytoplasmic membrane leaflets has not been observed in these species, but it has previously been described for another *Clostridium* species, *Clostridium nigrificans* (Sleytr et al. 1969). The expansion of the outer leaflet to 60–75 nm in thickness at the cell pole is an unusual phenomenon for a membrane lipid layer. The peptidoglycan layer is very thin (generally 2–3 nm), and layers this thin have been associated with a gram-negative staining reaction (Beveridge 1990). The identification of the S layer is based on direct comparison with other works on gram-variable cell walls (Sleytr et al. 1969; Sleytr and Glauert 1976). However, a regular array is visible in the S layer neither in thin-section nor by negative staining or shadowing. *Butyrivibrio fibrisolvens*, which exhibits a similar cell wall architecture, also possesses a smooth outer S layer (Beveridge 1990). This layer in *C. xylanolyticum* also does not appear to be effected by lysozyme and so is not part of the peptidoglycan of the wall. Thus, its location in the wall and its lack of peptidoglycan suggest it is an S layer.

The domed-cap expansion at the tip of the cell probably represents an accumulation of densely staining material such as protein or phospholipid; it is closely associated with the outer leaflet of the cytoplasmic membrane rather than being an expansion of the leaflet itself. The method of division exhibited by this bacterium is extremely unusual for a gram-positive bacterium, especially in light of the fact that a true septum is formed during sporulation. Vegetative division in gram-positive bacteria is by the process of septation (Beveridge 1981). Large numbers of cells have been seen displaying this phenomenon, and a septum has never been observed associated with any of these cells undergoing vegetative division.

The process of sporulation in *C. xylanolyticum*, while generally following the stages of sporogenesis as described by Ryter (1965), displays several aspects of ultrastructure that are distinctive. Many of these differences relate to the unusual structure of the cell envelope. Fitz-James and Young (1969) have studied sporogenesis in bacilli that give a gram-negative reaction and possess a multilayered cell envelope. According to this description, it is quite probable that these bacilli possess a cell envelope structure similar to that of *C. xylanolyticum*. How-

ever, those authors give no details, stating only that the processes of sporulation are very similar to those of gram-positive bacilli.

As is seen in sporogenesis in other endospore-forming bacteria, wall material is included in the spore septum. In *C. xylanolyticum*, the material is derived from the peptidoglycan layer of the wall. In other bacteria, this septum peptidoglycan is autolysed after septum formation and is laid down again after engulfment at the time of cortex formation (Illing and Errington 1991; Higgins and Piggot 1992). This removal of peptidoglycan means that the septum loses its rigidity and subsequently becomes very irregular in profile. Higgins and Piggot (1992) have suggested that with autolysis of the peptidoglycan there is a fusion of the two membranes and that during engulfment the prespore becomes surrounded by a single, bilayered membrane. They speculate that this membrane fusion and the associated loss of wall material relieves turgor pressure on the membrane to allow membrane movement and, thus, engulfment. The other membrane is laid down again when the peptidoglycan of the cortex is synthesized. This series of events is not seen in *C. xylanolyticum*, where the four electron-dense layers of the double membrane together with the central layer of wall material remain throughout the engulfment process. The engulfing membranes have a rigid appearance and never become irregular in outline. It is the peptidoglycan layer that imparts the mechanical strength to bacterial cell envelopes. It is therefore conceivable that the peptidoglycan derived from the cell wall is responsible for the observed rigidity of the developing spore membranes in *C. xylanlyticum.* However, since the layer is thin, it would allow enough flexibility for its movement during the engulfment process. Certain mutants of *Bacillus* have a thick peptidoglycan layer in their sporulation septum that is too stiff and thus prevents the engulfment from taking place (Oh et al. 1973). However, it is difficult to envisage how the mother cell nucleoid is partitioned and passes into the forespore with such a rigid septum, unless – in contrast to the process observed in other bacteria – it were to do so prior to the formation of the septum.

It has been observed that mesosomes are involved in prespore membrane development for several bacilli and clostridia (Fitz-James and Young 1969). They are associated with the cytoplasmic membrane at the point of septum formation and with the advancing membranes of the septum and, in bacilli, they also form in the central region of the spore septum. They follow the leading edge of the junction of the septum and the cytoplasmic membrane during engulfment. It has been shown using ultrarapid freezing together with freeze substitution and freeze fracture techniques that in several species of *Bacillus*, the mesosomes associated with septum formation are artifacts arising during conventional chemical electron microscopy preparation techniques (Ebersold et al. 1981a). The sporogenic vesicles observed in *C. xylanolyticum* appear as rigid structures possessing a very distinct bilayered wall and never exhibit an irregular outline as seen in mesosomes in other bacteria. Also, they are never associated with the cytoplasmic membranes. They are derived solely from wall material, i.e. they are peptidoglycan in origin. Because of this, they are enclosed within the advancing septum/wall junction. Therefore, they are not mesosomes at all, but some other structure; thus, here they have been given a distinct nomenclature, “sporogenic vesicles”.

During the engulfment process of bacterial sporogenesis, the junction of the septum and the cell envelope remains as a distinct entity that retains the same structure even as it moves towards the pole of the cell. In *C. xylanolyticum*, this region undergoes changes in morphology as the sporogenic vesicles form and then increase in number. They cause the two membranes of the septum in this region adjacent to the cell envelope to become widely separated. As the sporogenic vesicles move up along the sides of the prespore, they force these membranes even further apart. Thus, the junction of the septum with the cell envelope covers quite a distance rather than being a small discrete unit as seen in other bacteria. Only when the sporogenic vesicles disappear and engulfment is complete do these membranes come to lie adjacent to one another again.

In the seven-stage sporulation process of Ryter (1965), the cortex is formed in stage IV and the spore coat subsequently in stage V. This sequence of events has been observed during endospore sporogenesis in most bacilli and clostridia studied. However, in *C. xylanolyticum*, these events occur in reverse order. Spore coat fragments appear as early as stage II, and the spore coat forms a discontinuous layer prior to laying down of the cortex in stage V. The spore coat formation is not completed until after the deposition of the cortex. This sequence of events has also been observed in *Clostridium pasteurianum* (Santo et al. 1969). While the presence of spore coat fragments in stage II appears to be an early event, it has been shown that, in *Bacillus*, the synthesis of spore coat proteins commences in stage II (Aronson and Fitz-James 1976) although the spore coat is not visible ultrastructurally until stage V.

The persistence of the wall material in the engulfing prespore membrane has not been observed in endospore formation for other bacteria. However, in some cases, small, residual double-membrane regions have been observed in the septum after membrane fusion. It has been speculated that perhaps a small amount of wall material is retained associated with these and that it acts as the precursor for the cortex that is laid down after engulfment (Higgins and Piggot 1992). Typically in bacterial sporogenesis, the peptidoglycan that enters the spore septum is autolysed and new peptidoglycan is deposited in stage IV to form the cortex (Illing and Errington 1991; Higgins and Piggot 1992). The wall material in *C. xylanolyticum* is always readily visible during sporogenesis, persisting as a continuous layer between the membranes. This material subsequently expands to become the cortex. Thus, it is possible in *C. xylanolyticum* to trace the course of events that show that the cortex is a direct derivative of the cell wall.

The sporogenesis described here for *C. xylanolyticum* displays several unusual features. An ultrastructural study

utilizing cryofixation and cryosubstitution is necessary in order to further study these events. Additional ultrastructural detail has been revealed using these techniques to study other mature bacterial spores (Ebersold et al. 1981b). This type of study should also yield more information on the structure of the unusual cell envelope (Graham 1992).

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