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Ultrastructure of the Endotoxin Produced by Gram-negative Bacteria Associated with Organic Dusts

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Summary

The isolates of two out of four examined bacterial species associated with organic dusts (*Erwinia herbicola* and *Enterobacter* sp.) showed an ability to shed the membrane vesicles while growing on a solid medium. The formation of such vesicles was not observed in strains of other two species (*Acinetobacter calcoaceticus* and *Alcaligenes faecalis*) that revealed a lower degree of the outer membrane development. The preparations of crude lipopolysaccharide isolated with two methods from strains of all four species were found to be composed of vesicular particles resembling those shed from the outer membrane of *E. herbicola* and *Enterobacter* sp.

The similar structures were found in natural environment, using wood as a model substrate. It has been shown by electron microscopy and specific immunolabelling that wood dust from American basswood contained numerous gram-negative bacteria of the species *Erwinia herbicola* that were shedding large amounts of the endotoxin-containing membrane vesicles, measuring about 30 nm. Bacteria were not found in the samples of wood dust from other kinds of timber. These results were in full accord with those received by the examination of the wood dust samples by culture and by *Limulus* test for the presence of endotoxin.

The presented results suggest that the significant portion of an environmental "dust-borne" endotoxin occurs in the form of microvesicles, what may respresent an increased risk for the people exposed to various organic dusts.

Key words: Gram-negative bacteria – Endotoxin – Ultrastructure – Microvesicles – *Erwinia herbicola* – Wood dust

Introduction

The shedding of globular microvesicles from the outer membrane of gram-negative bacteria has attracted growing interest as a possible way of dissemination of hazardous microbial substances (endotoxin and other toxins, enzymes), mainly in relation to the oral environment and the etiology of periodontitis (*Nowotny* et al., 1982; *Mayrand* and *Grenier*, 1989).

So far the process of the formation of microvesicles has been observed only in liquid media (mainly broth , once in a tissue culture) (*Mayrand* and *Grenier*, 1989). Our preliminary studies (*Dutkiewicz* et al., 1988, 1989a) suggested that some of the gram-negative bacteria associated with organic dusts may release endotoxin mainly in form of vesicles and that the shedding of vesicles from the outer membrane occurred not only in liquid, but also in solid media and in native plant tissues.

These findings could be of importance for better understanding of the pathomechanism of occupational respiratory diseases (*organic dust toxic syndrome*, byssinosis) due to inhaling of organic dusts polluted with microbial products. It has been demonstrated that endotoxins produced by bacteria developing on grain, cotton, wood, straw and

other organic substrates play a significant role in etiology of these diseases (*Thelin* et al., 1984; *Rylander,* 1987; *Castellan* et al., 1987; *Jacobs,* 1989; *Lacey,* 1990), but so far it is not known in what physical form the endotoxin occurs in organic dusts, penetrates into lungs and exerts its damaging effects.

The aim of the present study was to verify these preliminary findings and to study a possibility of the formation of microvesicles by the strains of gram-negative bacteria growing on a solid substrate and by the strains naturally occurring in plant tissues, using wood samples as a model substrate. To better understand this process, the ultrastructure of the outer membrane in the isolates of four species of gram-negative bacteria, representing major dust contaminants in various working environments polluted with organic dusts (*Dutkiewicz,* 1978, 1986, 1989; *Rylander* and *Lundholm,* 1978; *Clark* et al., 1983; *Lacey,* 1990) was compared with the ultrastructure of the endotoxin (lipopolysaccharide, LPS) derived from the same isolates.

Materials and Methods

Bacterial strains. The strains were: *Erwinia herbicola* (synonyms: *Enterobacter agglomerans, Pantoea agglomerans*): strain M-10-3 isolated from the air of a grain mill in the region of Lublin, Poland; *Erwinia herbicola:* strain WD-72 isolated from a wood sample taken from American basswood in West Virginia, USA; *Enterobacter* sp.: strain TR-2 isolated from the air of a sawmill processing pine wood in the region of Lublin; *Acinetobacter calcoaceticus* var. *lwoffi:* strain WP-6 isolated from the air of a poultry hatchery in the region of Lublin; and *Alcaligenes faecalis:* strain HB-11 isolated from the air of a herb processing plant in Lublin.

Ultrastructure of the cell envelope. Bacteria were grown on nutrient agar slants for 48 h at 34 °C. Small portions of bacterial mass were picked with a loop, pre-fixed for 2 h in 2% glutaraldehyde in phosphate buffer at pH 7.3, rinsed overnight in 7% sucrose in phosphate buffer at pH 7.3, and then post-fixed in 1% buffered osmium tetroxide for 1.5 h. After dehydration in graded series of ethanol, the samples were embedded in Epon 812, thin sectioned (silver colour) and post-stained with 2% uranyl acetate and lead citrate.

All micrographs were taken with a Philips EM 300 electron microscope operating at 80 KV. The size of observed particles was determined from photomicrographs. Fifty measurements of the long axis diameter were done for each sample.

The ultrastructure of the *Erwinia herbicola* strain WD-72 from wood was examined by slightly different methods. The bacteria were grown on tryptic soya agar slants for 72 h at 34 °C, and then were prepared, thin-sectioned and viewed as described for the wood samples (see below).

Extraction of LPS. Extraction was made from all strains except for the WD-72 strain of *E. herbicola.* Bacteria were propagated on nutrient agar in Roux bottles for 48 h at 34 °C. The lipopolysaccharide was derived from the washed bacterial mass of each strain by two methods: 1) The trichloroacetic (TCA) method of *Boivin* et al. (1933), consisted of extraction with 0.25 M TCA, dialysis against water, precipitation with acetone and final lyophilization. 2) The phenol-water (P-W) method of *Westphal* et al. (1952), consisted of extraction with 45% phenol, separation of the water phase, dialysis against water and final lyophilization.

Ultrastructure of LPS. Samples of the P-W extract of *E. herbicola* (strain M-10-3) and of the TCA extract of *A. calcoaceticus* were studied after thin-sectioning. Small portions of the lyophilized LPS powders were prepared, thin-sectioned and viewed in the same manner as whole bacterial cells.

All the TCA and P-W extracts from the four strains were studied directly by negative staining. Samples of lyophilized lipopolysaccharide were resuspended in distilled water and stained on grids with 2% aqueous uranyl acetate for 5 seconds.

Wood samples. Samples were taken in August and October 1987 from the sapwood of stored timber logs of American basswood (*Tilia americana* L.), red oak (*Quercus coccinea* Muenchh.) and white poplar (*Populus alba* L.). The logs had been stored for a period of 4–6 weeks before sampling in a lumber yard at a sawmill in West Virginia, USA. The wood surface to be sampled was disinfected by wiping with 70% propanol and a 5.25% sodium hypochlorite solution. Samples were taken with a novel drilling device that collects the pulverized wood into a flask attached beneath the bit in a one-step sterile process (*Dutkiewicz* et al., 1989b).

Estimation of the numbers of gram-negative bacteria in wood. Viable counts of gram-negative bacteria were determined by dilution plating. Wood samples (200 mg) were suspended in 20 ml of sterile phosphate buffered saline containing 0.1% (v/v) Tween 80 and, after vigorous shaking, serial 10-fold dilutions were made up to 10^{-6}. 0.1 ml of each dilution was spread on duplicate sets of eosin methylene blue agar plates (EMB agar, Difco) and incubated for 48 h at 35 °C. Gram-negative isolates were subcultured on tryptic soya agar (Difco) slants and identified with API kits 20 E (for enterobacteria) and Rapid NFT (for non-fermenting bacteria). Results were reported as colony forming units (CFU) per gram of pulverized wood.

Estimation of the concentration of endotoxin in wood. Wood samples (100 mg) were extracted with 5 ml of sterile non-pyrogenic water by rocking for 1 h at room temperature. The suspension was centrifuged at 1000 g for 10 min to remove particulate debris. Quantification of gram-negative bacterial endotoxin in the aqueous extract was performed in duplicate by a chromogenic modification of the *Limulus* amoebocyte lysate test (QCL-1000; Whittaker Bioproducts, Walkersville, MA). Results were reported as endotoxin units (EU) per gram of pulverized wood.

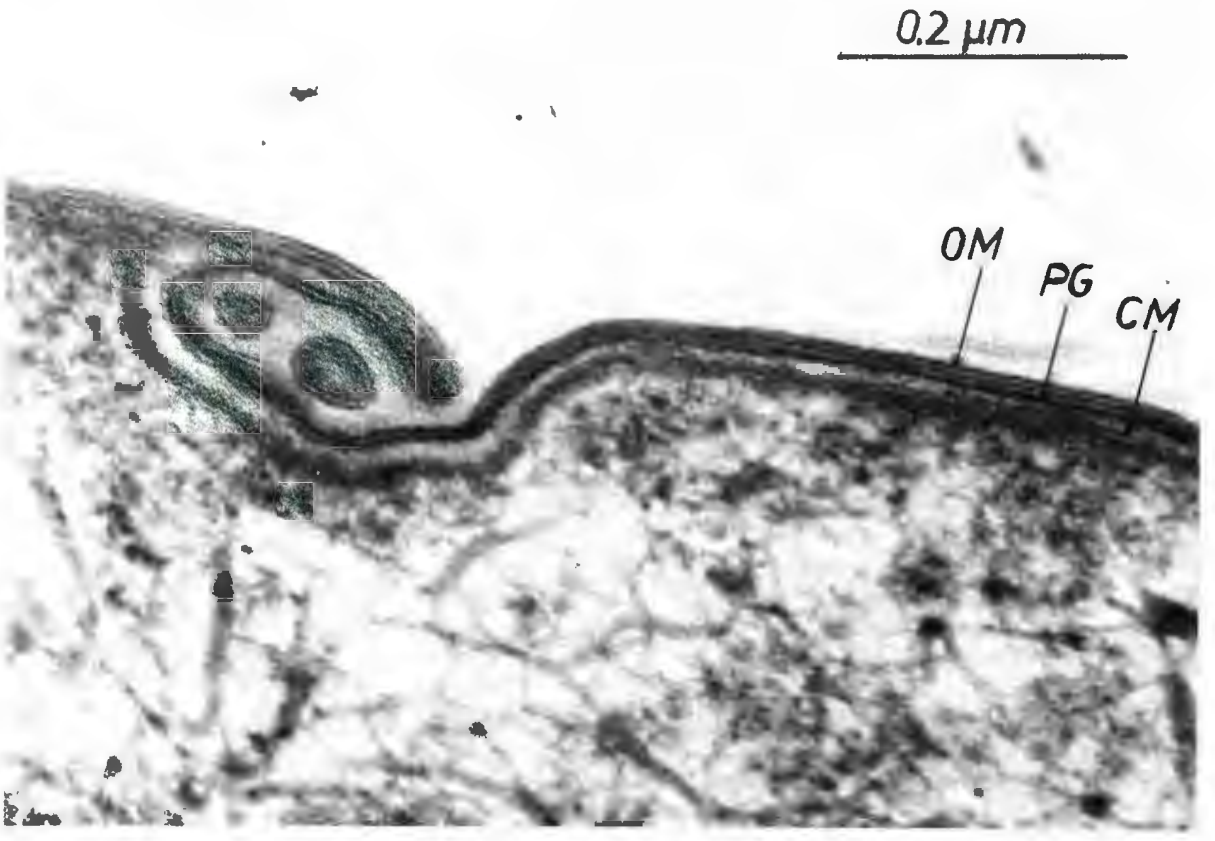
Ultrastructural examination of wood samples. Small portions of pulverized wood (circa 20 mg) were pre-fixed for 3 h in 3.0% Karnovsky's fixative (*Karnovsky,* 1965) in 0.1 M Sorensen's phosphate buffer at pH 7.4, then placed for 1 h in 2.0% tannic acid, and post-fixed for 1 h with 2.0% osmium tetroxide. After dehydration in graded series of ethanol, the samples were embedded in Epon LX 112, thin-sectioned (silver colour), mounted on copper grids, and stained with 4% uranyl acetate in 50% ethanol and Reynolds lead citrate.

All micrographs were taken with a JEOL 100 CX electron microscope operating at 80 KV. The size of observed particles was determined from photomicrographs. Fifty measurements of the long axis diameter wer done for each sample.

Hyperimmune sera. For immunological identification of the observed structures, antisera against cell wall lipopolysaccharide (LPS) and lipid A (LA) antigens of *Erwinia herbicola,* strain M-10-3 were raised in rabbits. Strain M-10-3 has been used previously as a standard strain for these purposes (*Dutkiewicz,* 1978).

LPS antigen was obtained by the TCA method of *Boivin* et al. (1933) as described above. Rabbits were injected intraveneously (i.v.) 5 times on every second day with increasing doses of LPS equal to 20 μg, 40 μg, 60 μg, 80 μg and 100 μg, and then received booster doses of 100 μg on 7th and 17th day after the end of primary immunization.

Lipid A antigen was prepared according to the method of *Galanos* et al. (1971), comprising treatment of cell mass with 1%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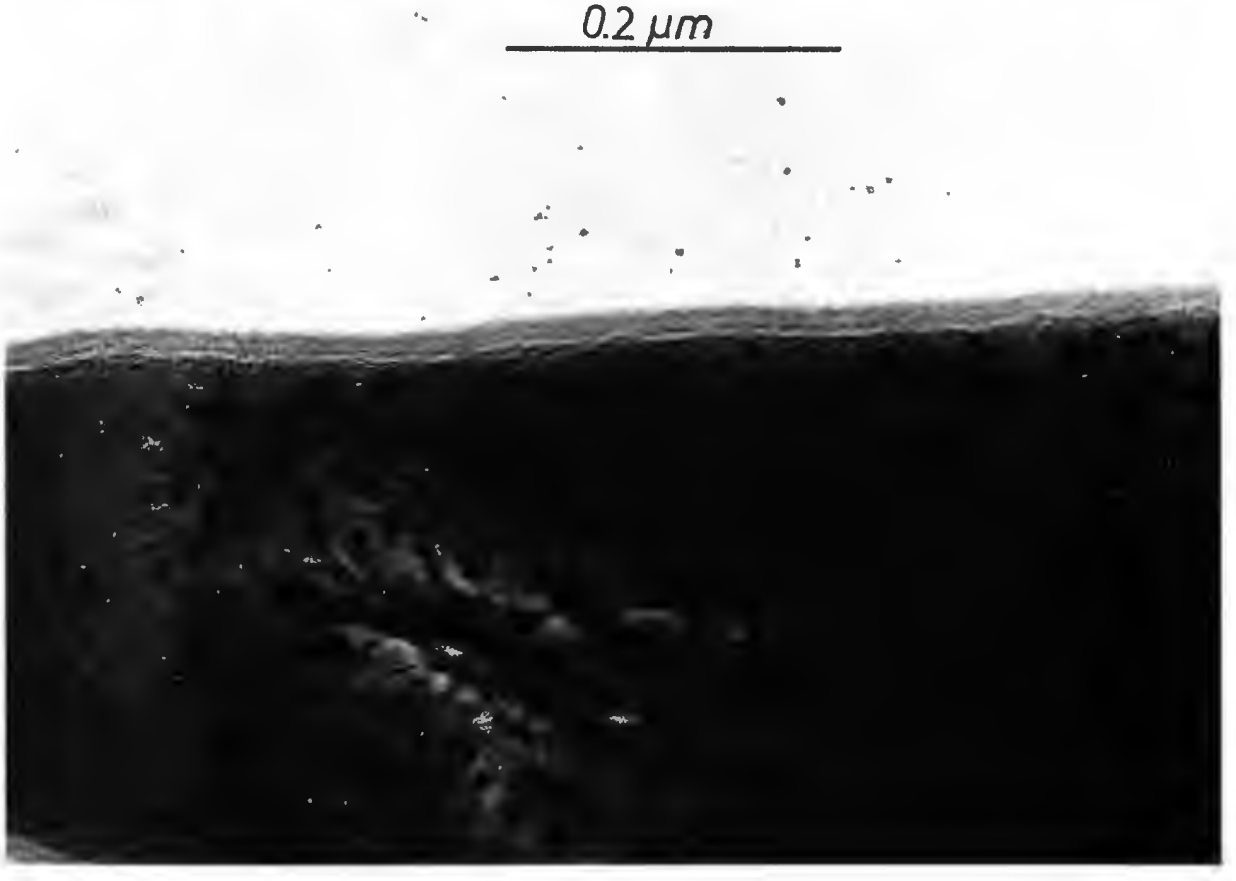
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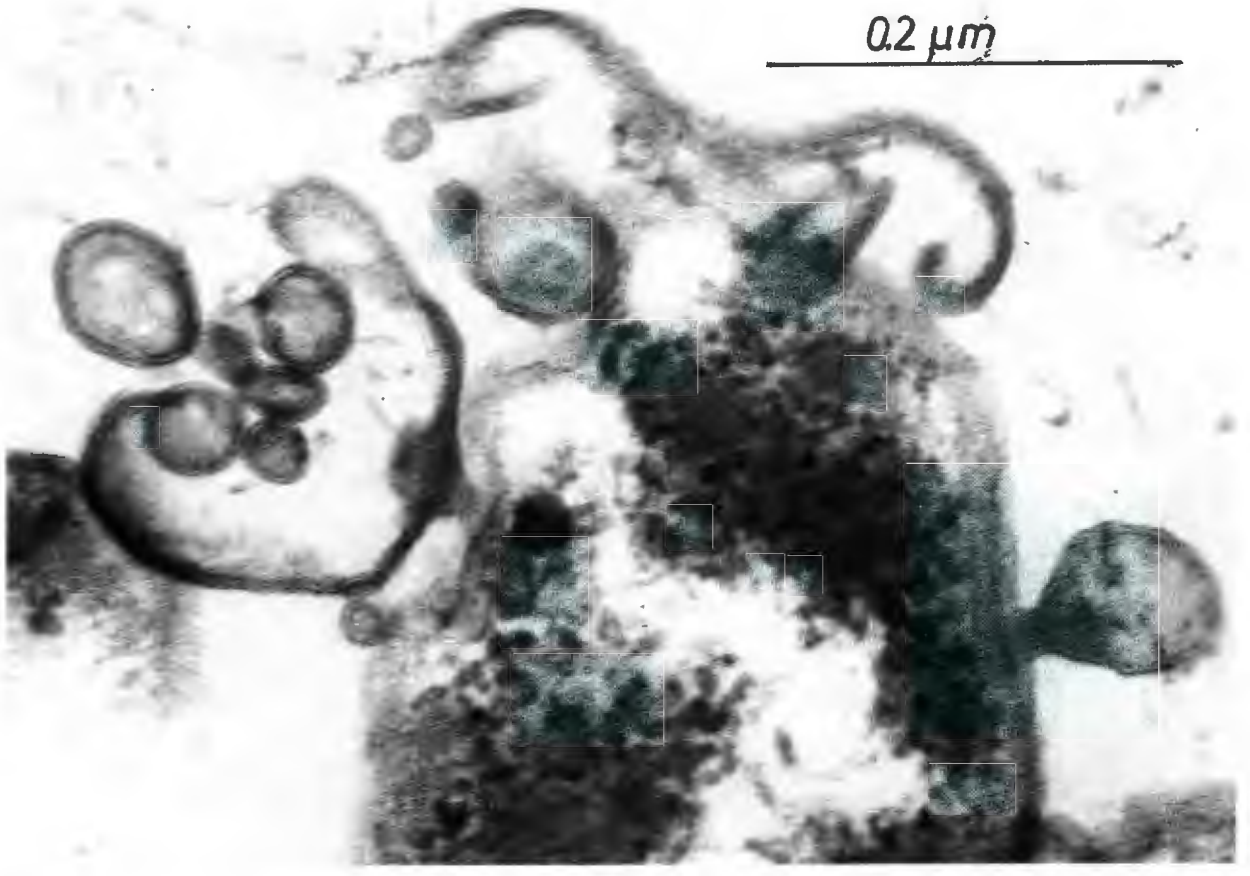


Fig. 3. Thin-sectioned cell of *Erwinia herbicola* (strain WD-72, agar culture) showing formation of vesicles from outer membrane by a budding-like process. Note structures suggesting formation of smaller blebs inside larger ones by internal budding.

acetic acid followed by triethylamine extraction. Polyclonal antiserum was produced in mice according to an immunization schedule used by *Miner* et al. (1986).

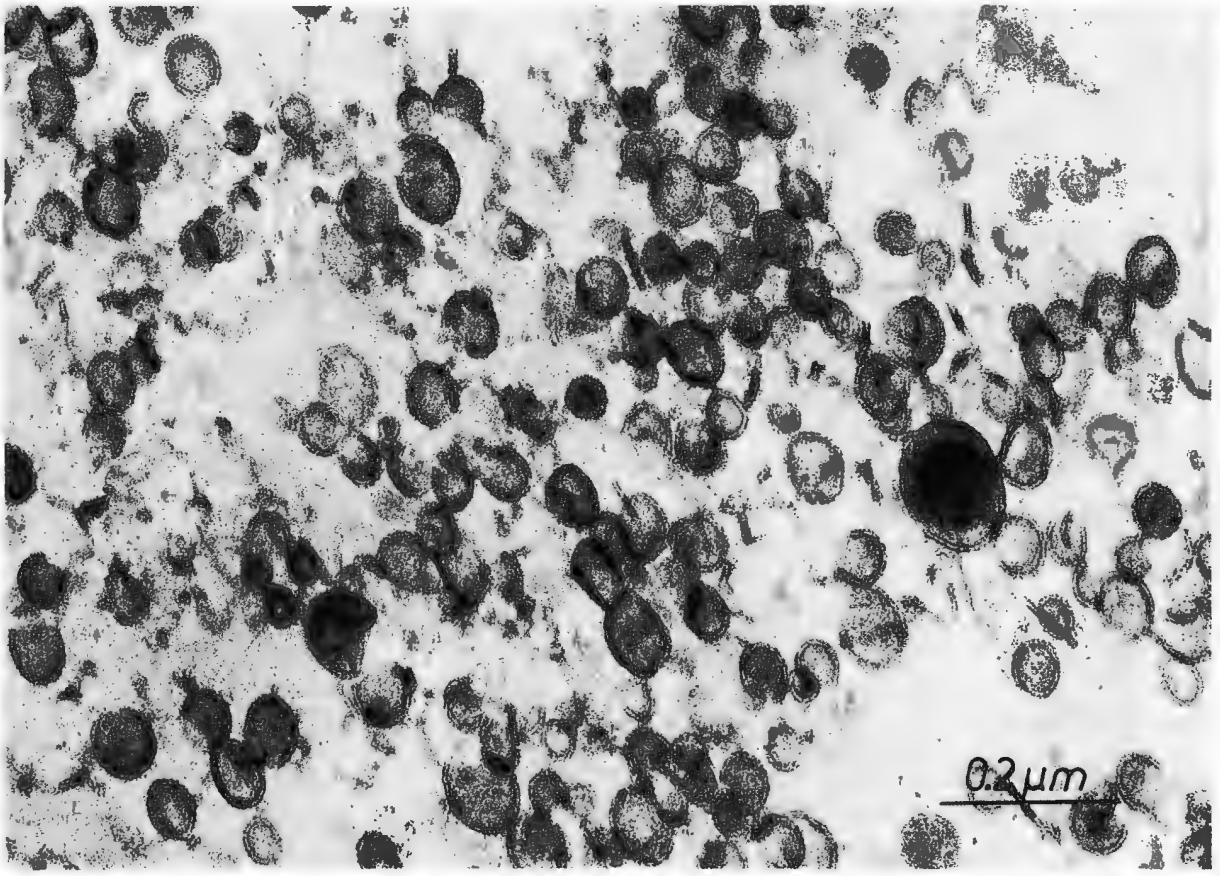
The received anti-LPS and anti-LA sera were tested by ELISA test with the antigen of *E. herbicola* M-10-3. The sera showed positive reactions up to a titre of 1 : 2560.

Staining with immunogold. The post-embedding "on grid" labelling technique was used. Samples of pulverized wood and of the cell mass of the *Erwinia herbicola* strain WD-72 were fixed for 2 h in 2.5% glutaraldehyde, dehydrated in graded series of ethanol, and embedded in LR White medium (Polysciences Inc., Warrington, PA). The samples were thin-sectioned (100–150 nm) and mounted on nickel grids.

A buffer (TBT), consisting of 20 mM Tris buffered saline (TBS) supplemented with 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 (T-20), was used for washing the grids (pH adjusted to 7.2) and for diluting the antisera and immunogold reagent (pH adjusted to 8.1). The grids were washed in TBT twice for 5 min, floated in the hyperimmune sera against LPS or LA of *E. herbicola* diluted 1 : 30 for 2 h, washed again in TBT twice for 5 min, floated in the conjugate of colloidal gold (particle size 5 nm) with goat anti-rabbit IgG or goat anti-mouse IgG+IgM (Janssen Life Sciences Products, Piscataway, NJ) diluted 1 : 20 for 1 h, and finally washed in TBT twice for 5 min and in distilled and demineralized water twice for 5 min. Grids were dried on filter paper, left unstained or post-stained with 4% uranyl acetate in water and Reynolds lead citrate, and examined in the JEOL 100 CX electron microscope as above. The density of labelling was measured from photomicrographs by quantitative morphometry and was expressed as the numbers of particles/cm^2.

Fig. 1. Thin section of the cell envelope of *Erwinia herbicola* (strain M-10-3, agar culture), showing clearly three layers: outer membrane (OM), peptidoglycan layer (PG) and cytoplasmic membrane (CM). The OM has a very distinct trilaminar structure. Inside a pouch created by convolution of the cell envelope are seen two membrane vesicles showing the morphology identical with that of thin-sectioned LPS particles (see Fig. 4).

Fig. 2. Thin section of the cell envelope of *Alcaligenes faecalis* (strain HB-11, agar culture). Neither clear differentiation into separate layers nor a trilaminar structure of OM can be seen. The electronically dense material at the surface may be related to the presence of lipopolysaccharide.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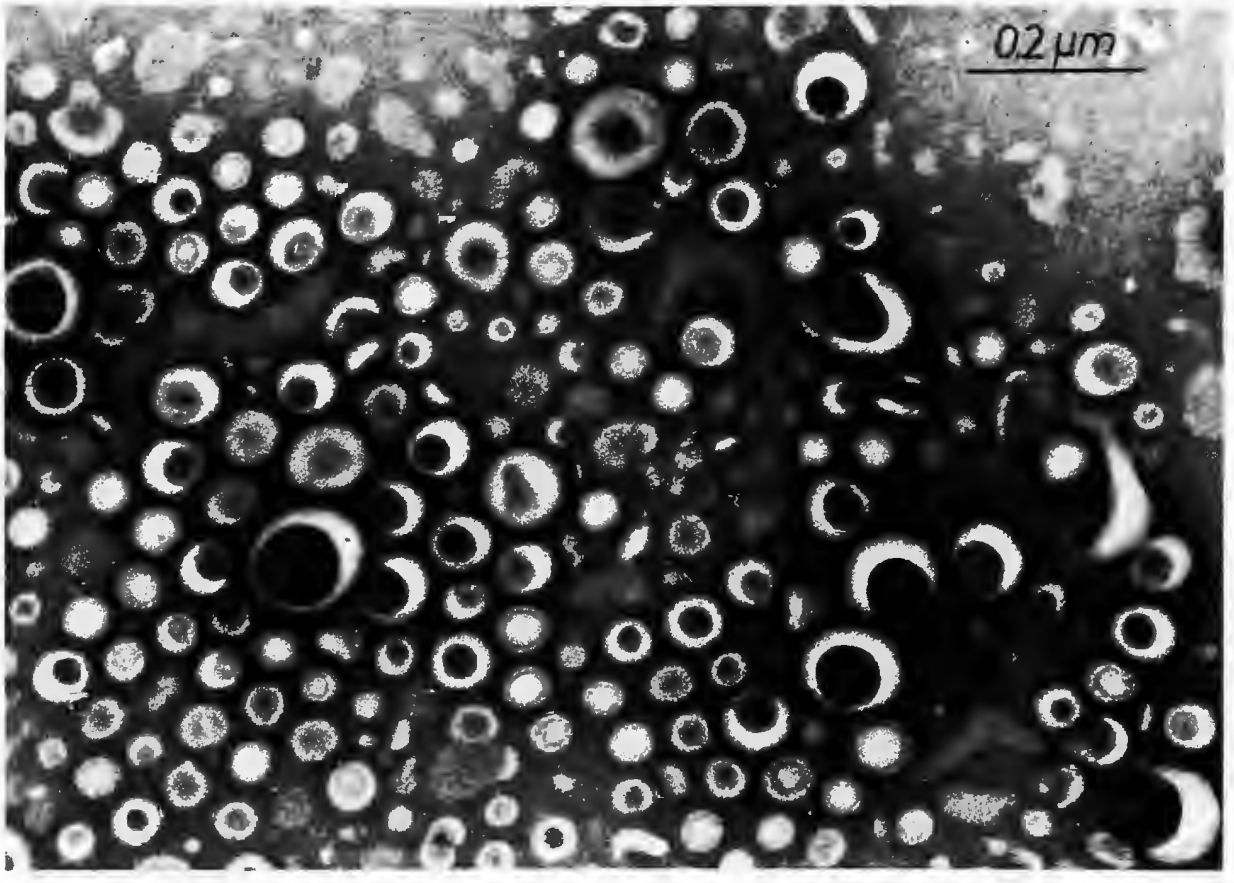
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Table 1. Gram-negative bacteria and endotoxin in the particular kinds of woods

Wood	Total count of gram-negative bacteria (CFU × 10^3 per gram)		Count of *Erwinia herbicola* (CFU × 10^3 per gram)		Concentration of bacterial endotoxin (EU × 10^3 per gram)		Ultrastructural evidence for gram-negative bacteria and endotoxin		Immunogold-labelling for LPS of *Erwinia herbicola*		Immunogold-labelling for lipid A of *Erwinia herbicola*	
	A[a]	B[b]	A	B	A	B	A	B	A	B	A	B
American basswood	300.0	218.0	300.0	54.9[c]	182.47	280.40	Pos.[d]	Pos.	Pos.	Pos.	Pos.	Pos.
Red oak	0	0	0	0	0.14	0.34	Neg.[e]	Neg.	Neg.	Neg.	Neg.	Neg.
White poplar	0	0	0	0	0.12	0.19	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

[a] A = August. [b] B = October. [c] Associated gram-negative bacteria were: *Klebsiella* sp., *Citrobacter freundii* and *Pseudomonas putida*. [d] Pos. = Positive. [e] Neg. = Negative.

Results

Cell envelopes of agar-cultured bacteria

The cell envelopes of the two enterobacterial strains (*Erwinia herbicola* and *Enterobacter* sp.) were clearly differentiated into an outer membrane, a peptidoglycan layer, and the cytoplasmic membrane. The outer membrane of both showed a very distinct trilaminar (triple-tracked) structure (Fig. 1). In two non-enterobacterial species (*Acinetobacter calcoaceticus, Alcaligenes faecalis*) the degree of development of the cell envelope appeared to be less, particularly in *A. faecalis* (Fig. 2).

Shedding of membrane vesicles by agar-cultured bacteria

Outside the cell envelope of bacteria belonging to the *Erwinia herbicola* strains M-10-3 and WD-72 were seen membrane vesicles (Figs. 1 and 3) measuring on the average ($\bar{x} \pm$ S.D.) 44.7 ± 17.7 nm and 36.5 ± 16.8 nm, respectively. Inspection of Figs. 1 and 3 suggest that the vesicles may be formed in the course of a process involving convolution of the outer membrane followed by a stage resembling "internal budding". Shedding of vesicular particles from the outer membrane was observed also in cultures of the *Enterobacter* sp., but to a lesser extent than in *E. herbicola*. Shedding was not observed in the cultures of *A. calcoaceticus* and *A. faecalis*.

Isolated endotoxin

Thin-sectioned crude LPS preparations from the *E. herbicola* and *A. calcoaceticus* strains were found to be composed of vesicular particles (Fig. 4) showing identical morphology to those shed from the outer membrane of *E. herbicola* (Figs. 1 and 3). They had a characteristic, 4–5 nm wide margin and their global shape strongly resembled broken nut shells.

A similar picture of the endotoxin was found in the directly stained crude LPS preparations from all the strains examined. It appeared as loose aggregations of shell-like, vesicular particles (Fig. 5). The LPS particles from the different strains showed a high degree of similarity, irrespective of the method of extraction.

The mean long axis diameters of the particles found in the ten examined LPS preparations ($\bar{x} \pm$ S.D.) ranged from 35.9 ± 12.5 nm to 82.7 ± 26.3 nm.

Occurrence of gram-negative bacteria and endotoxin in wood samples

High levels of viable gram-negative bacteria and endotoxin were found in the pulverized samples from American basswood. The red oak and white poplar samples contained no viable bacteria and only trace amounts of endotoxin (Table 1). Electron microscopic examination revealed structures morphologically similar to gram-negative bacteria and endotoxin in the basswood samples. These showed positive reactions with antisera against the LPS and lipid A of *Erwinia herbicola*, the dominant gram-negative bacterium in the wood (Table 1). No such structures were found in the red oak or white poplar wood.

Ultrastructure of gram-negative bacteria and endotoxin in wood samples

The structures clearly resembling those seen in the agar cultures of *Erwinia herbicola* were observed in thin-sectioned samples of the pulverized wood from basswood (Figs. 6, 7 and 8). The bacteria were seen usually in the lumen of lignified wood cells (vessels or fibres) (Fig. 6). The membrane vesicles appeared to be peeling off the out-

Fig. 4. Thin-sectioned particles of the isolated lipopolysaccharide (LPS) from *Erwinia herbicola* (strain M-10-3, P-W extract).

Fig. 5. Lipopolysaccharide (LPS) from *Enterobacter* sp. (strain TR-2, TCA extract), negatively stained with uranyl acetate.

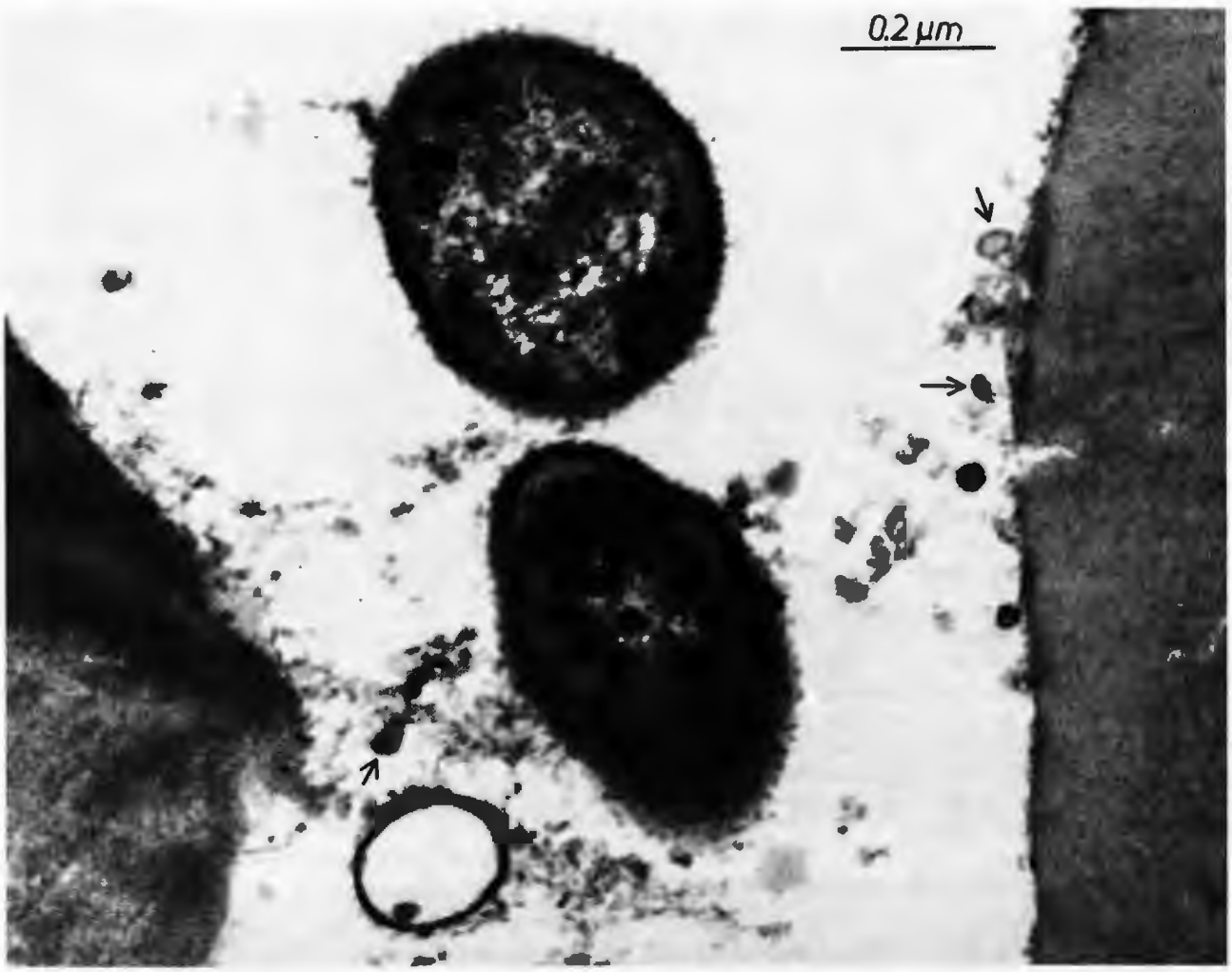


Fig. 6. Thin-sectioned sample of pulverized wood from American basswood (taken in August 1987) showing two cells of gram-negative bacteria and numerous membrane vesicles (marked with arrows) in the lumen of a wood cell.

er membranes of bacteria (Figs. 6 and 7) and occurred, singly or in aggregates, in the lumen of wood cells. Some free vesicles adhered to the inner layer coating the lumen of lignified cells (Fig. 6). Abundant release of the membrane vesicles was observed in deteriorating bacterial cells (Fig. 8). The free vesicles seen in basswood samples measured on the average 29.5 ± 10.1 nm.

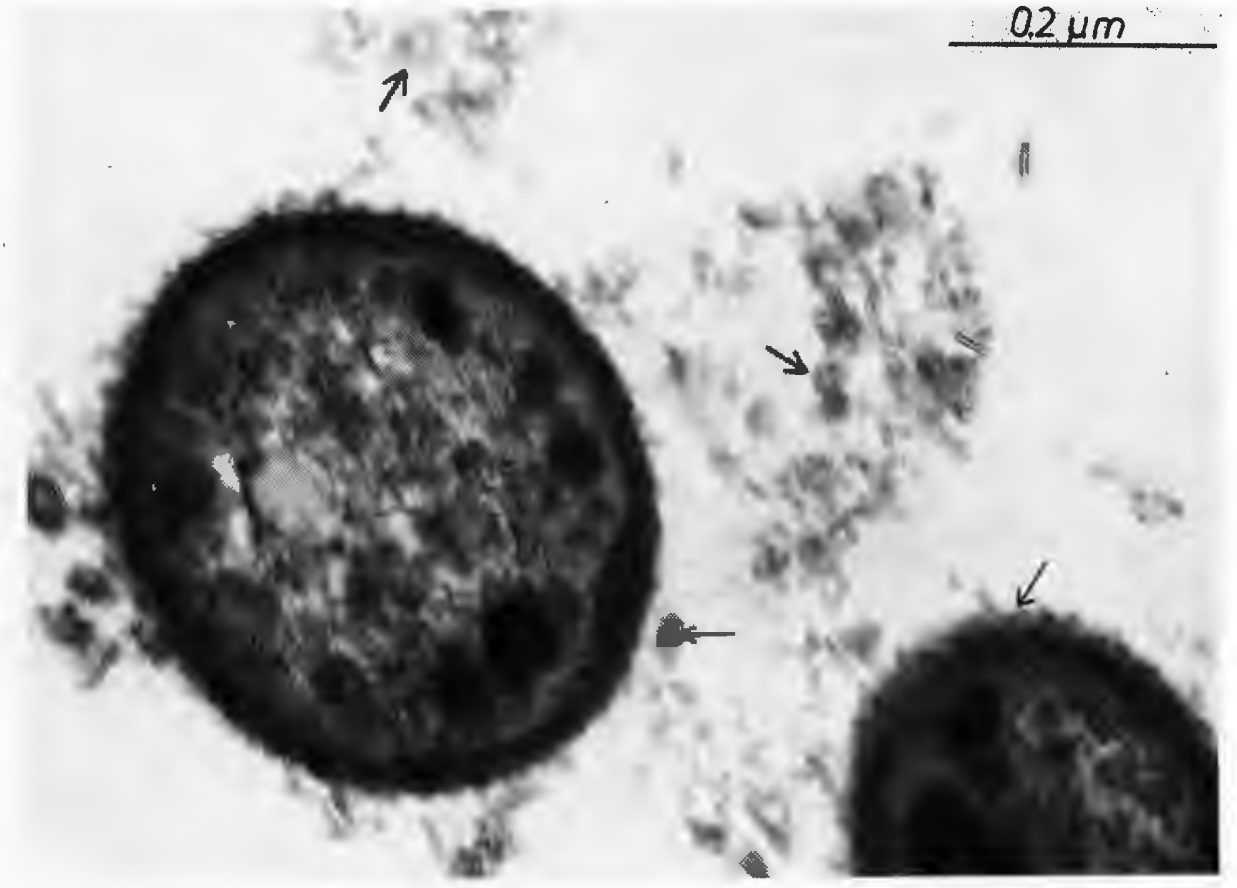
Reaction with specific antibody

Whole cells of the *Erwinia herbicola* strain isolated from the basswood reacted positively with specific anti-LPS and anti-lipid A antibodies, as shown by the deposition of immunogold particles in the outer membranes. The arrangement of gold particles in the form of cones protruding from the outer membrane or as circles outside the membrane (Fig. 9), corresponded to the previously observed shapes of the vesicles released from the bacterial cell envelopes. This suggests that these vesicles are in fact supermacromolecules of endotoxin.

A similar arrangement of immunogold particles around structures corresponding to bacteria and membrane vesicles was observed in thin-sectioned samples of pulverized basswood (Fig. 10). The density of gold particles on these structures was five fold greater than the background. Gold particles were also seen in similarly treated samples of red

Fig. 7. Thin-sectioned sample of pulverized wood from American basswood (taken in August 1987). Peeling of the membrane vesicles (marked with arrows) from the outer membrane of gram-negative bacteria is clearly seen.

Fig. 8. Thin-sectioned sample of pulverized wood from American basswood (taken in August 1987) showing structures corresponding to the deteriorated cells of gram-negative bacteria, bearing numerous membrane vesicles (marked with arrows).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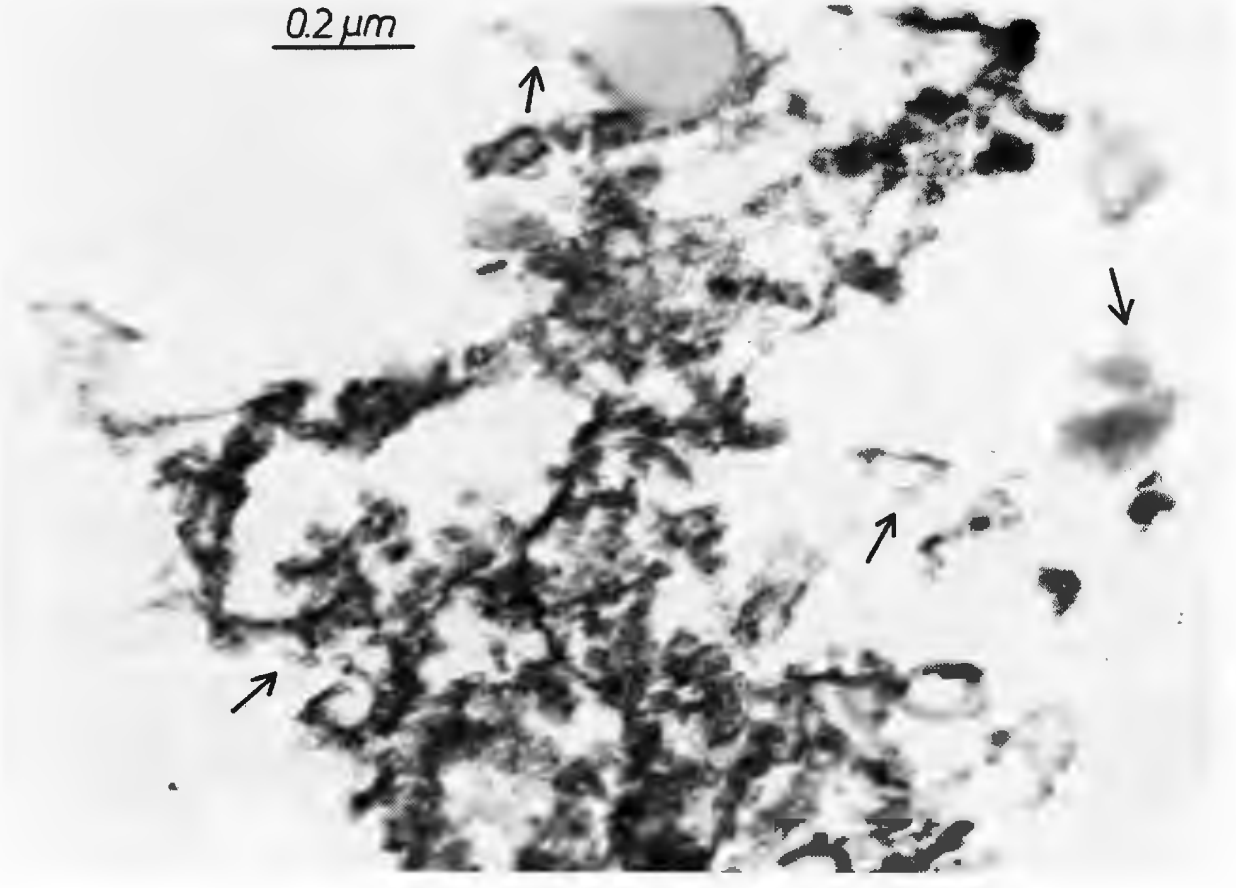
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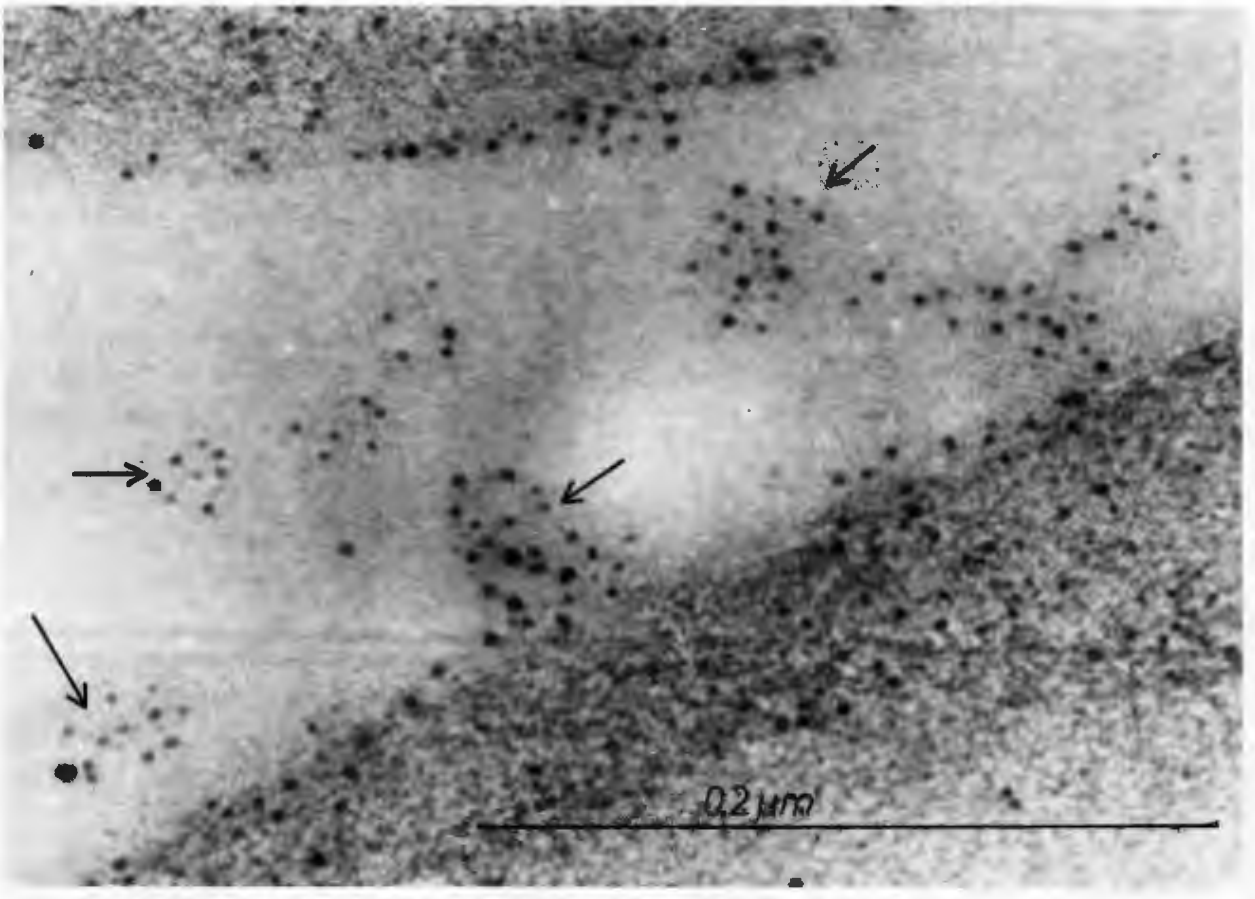

Fig. 9. Thin-sectioned cells of *Erwinia herbicola* (strain WD-72, agar culture) immunostained with rabbit antiserum against LPS of *E. herbicola* and gold-labelled with anti-rabbit IgG. Arrows show aggregations of gold particles upon structures corresponding in shape and size to vesicles budding from outer membrane.

oak and white poplar wood but in these cases they were evenly distributed over wood tissue with no affinity to particular structures.

Discussion

The cells of the strains of two enterobacterial species associated with organic dusts (*Erwinia herbicola, Enterobacter* sp.) showed an ability to shed membrane vesicles when grown on a agar medium. Shedding of similar vesicles has been reported by other workers in strains of a number of gram-negative genera (*Work* et al., 1966; *Nowotny* et al., 1982; *Lounatmaa* and *Helander,* 1982; *Freer,* 1985; *Mayrand* and *Grenier,* 1989). However, all these cultures were grown in liquid media. To the best of our knowledge the present paper is the first to report shedding of membrane vesicles by bacteria grown on a solid medium.

We observed that the membrane vesicles are formed by a two-step process involving convolution of the outer membrane followed by a budding-like detachment of the terminal blebs. The process might proceed not only as external budding, as described by *Mayrand* and *Grenier* (1989) but also as internal budding into a pouch-like structures. As the vesicles closely resembled the particles seen in preparations of the isolated crude lipopolysaccharide and showed a positive reaction with specific anti-LPS and anti-Lipid A immune sera they can be considered as endotoxin-containing supermacromolecules.

The formation of membrane vesicles was not observed in the strains of the two non-enterobacterial species (*A. calcoaceticus* and *A. faecalis*) that revealed a low degree of outer membrane development. It cannot be excluded, however, that this process may occur also in these species that are able to produce biologically active endotoxins (*Dutkiewicz* et al., 1988), but was not intensive enough to be recorded by us.

The ultrastructure of the outer membrane and/or the endotoxin of *Acinetobacter calcoaceticus* and *Erwinia herbicola* have been examined by other workers (*Thornley* and *Glauert,* 1968; *Symons* et al., 1979; *Helander* and *Lounatmaa,* 1981). We were unable to find any such studies on *Alcaligenes faecalis* or *Enterobacter* sp. associated with pine wood. *Thornley* and *Glauert* (1968) did not describe shedding of membrane vesicles by *Acinetobacter calcoaceticus* but observed them after digestion of cell envelope with enzymes. We never observed shedding of rib-

bon-like structures reported by *Helander* and *Lounatmaa* (1981) in broth cultures of *Erwinia herbicola* and of other bacteria associated with cotton.

The physical shape of endotoxin, referred to by *Burrell* (1990) as "the entire LPS complex in association with the naturally occurring proteins that make up the structural integument of Gram-negative bacteria", is still a subject of controversy. Endotoxin macromolecules are described mostly as long ribbon-like structures or as globular bodies (*Shands*, 1971). Based on the examination of preparations of both the outer mebrane and the isolated crude lipopolysaccharide our results suggest that the typical shape of the endotoxin supermacromolecule produced by some gram-negative bacteria associated with organic dusts is a vesicular, shell-like particle measuring 30–50 nm.

Very similar structures were found in natural environment, using wood as a model substrate. The results of the electron microscopic and specific immunolabelling studies indicate that wood dust from the American basswood contained the cells of *Erwinia herbicola* that were shedding large amounts of the endotoxin-containing membrane vesicles clearly resembling both the microvesicles shed by the agar-cultured bacteria and the particles of isolated lipopolysaccharide. Bacteria were not detected in the samples of wood dust from other kinds of timber (red oak, white poplar). These results are in accord with those obtained when wood dust samples were examined by dilution plating and by the *Limulus* test.

Free endotoxin-containing membrane vesicles have been described previously only in cultures of various gram-negative bacteria (*Mayrand* and *Grenier*, 1989), not in the natural environment. Biologically active endotoxin has been detected by the *Limulus* test in different natural environments, such as water (*DiLuzio* and *Friedmann*, 1973), plant materials (*Rylander* and *Lundholm*, 1978) and organic dusts (*Rylander* and *Lundholm*, 1978; *Dutkiewicz*, 1986), however, the typical physical form of the free endotoxin remained unknown (*Burrell*, 1988). To the best of our knowledge, this study presents the first visualization of environmental endotoxin. It appears to be an assemblage of discoid vesicles (globules) peeling off the outer membrane of the bacteria. The vesicles have a characteris-

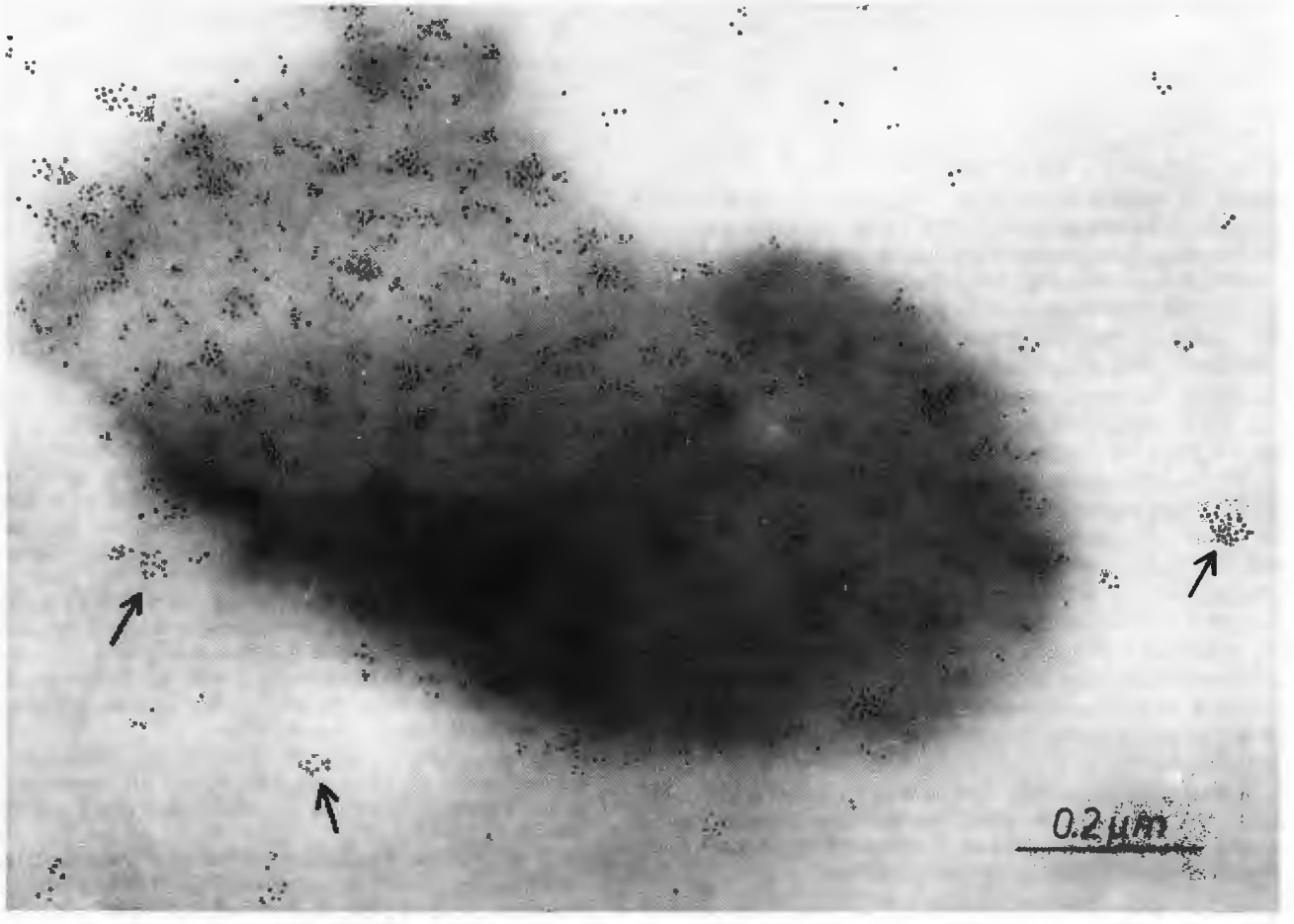

Fig. 10. Thin-sectioned sample of pulverized wood from American basswood (taken in August 1987) immunostained with the rabbit antiserum against LPS of *Erwinia herbicola* and gold-labelled with anti-rabbit IgG. Structure corresponding to the cell of gram-negative bacterium is seen, stained positively with immunogold. Arrows outside the cell show aggregations of gold particles to smaller structures corresponding in shape and size to membrane vesicles.

tic "tripled-tracked" membrane (dark-light-dark), corresponding to the structure of the outer membrane of bacterial cell. The photomicrographs indicate that these vesicles are formed by a budding-like convolution of outer membrane and are abundantly released from bacterial cells in wood tissue. The specific immunolabelling with colloidal gold showed that the vesicles contain lipopolysaccharide and its constituent lipid A.

Wood is recognised as one of the sources of offending organic dusts, that cause respiratory disorders (*organic dust toxic syndrome*, allergic alveolitis, asthma, rhinitis) in the exposed workers (*Goldsmith* and *Shy*, 1988). Wood inhabiting microorganisms, in particular fungi and endotoxin-producing gram-negative bacteria, have been implicated as the putative agents of the disease (*Wilhelmsson* et al., 1984; *Kolmodin-Hedman* et al., 1987; *Dutkiewicz*, 1989). It is known, that gram-negative bacteria may develop abundantly in stored wood (*Rossell* et al., 1973).

Erwinia herbicola has been implicated as a main source of potentially pathogenic endotoxin in cotton dust (*Rylander* and *Lundholm*, 1978; *Rylander*, 1987) and grain dust (*Dutkiewicz*, 1976). Our data indicate that it may play a similar role in wood dust. If so, the exposure to dust from particular kinds of woods (e.g. American basswood) may carry a risk of the endotoxin-induced acute respiratory disorders in the workers of sawmills and other wood processing facilities, similar to those observed in cotton workers (*Rylander*, 1987).

The observation that a significant portion of the environmental "dust-borne" endotoxin occurs in the form of globules (vesicles) measuring 10–50 nm is in accord with results of *Rylander* et al. (1989) who found a peak of endotoxic activity in the smallest fraction (below 0.5 μm) of cotton dust particles. The microvesicular shape of dust-borne endotoxin represents a particular risk as a respiratory hazard, because of the particular ease with which particles of this shape and size interact with alveolar macrophages and other immune cells (*Burrell*, 1988; *Özel* et al., 1988), to initiate inflammatory processes in the lungs (*Rylander*, 1987; *Burrell* 1990).

The results confirm the view (*Rylander*, 1988; *Rylander* et al., 1989; *Burrell*, 1988, 1990) that studies on the effect of inhaled dust-borne endotoxin on lung function should not be performed with chemically purified lipopolysaccharide or lipid A but rather with a fraction of disrupted bacterial cells more closely resembling the appearance of the endotoxin complex in the natural environment. The microvesicle fraction is particularly suited to this purpose as it can be separated easily in a sucrose gradient (*Burrell* et al., in preparation).

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