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Envelope glycosylation determined by lectins in microscopy sections of *Acinetobacter venetianus* induced by diesel fuel

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Abstract

It was suggested in a previous study that cells of *Acinetobacter venetianus* VE-C3 adhere to diesel fuel by synthesizing a capsular polysaccharide containing glucose and/or mannose. To study the fine structure of cells and localization of bacterial polysaccharide in the presence of diesel fuel, two lectins were used: ConA, an agglutinin from *Canavalia ensiformis* specific for mannose and/or glucose residues, and PNA, an agglutinin from *Arachis hypogaea*, for terminal galactose residues. The lectins were conjugated with electron dense ferritin for transmission electron microscopy (TEM) and with fluorescein isothiocyanate (FITC) for scanning confocal laser microscopy (SCLM). Samples were prepared by freeze substitution, which allows glycosylation to be determined in situ in thin sections of specimens. The distribution of glycosylation was imaged with and without treatment of specimens with their specific hapten (glucose and galactose). The glycosylation activity produced a polysaccharide capsule. Emulsified diesel fuel nanodroplets were observed at the cell envelope perimeter. Fine structure of vesicles consisted of polysaccharide and diesel fuel nanodroplets. Lectin blotting analysis showed ConA-positive glycoprotein with an apparent molecular mass of 22 kDa in the outer membrane. Its production was induced by diesel fuel. This glycoprotein was probably responsible for bioemulsifying activity at the cell envelope. Several other glycoproteins were positive for PNA lectin, the main constituent migrating with an apparent molecular weight of 17.8 kDa. However, they were all constitutive and probably involved in cell biofilm formation at the oil surface.

Keywords: Glycosylation; Capsule; Composite material; Glycoprotein; Lectin blotting; Biofilm; Microscopy; TEM; SCLM

1. Introduction

The ability of microbes to utilize hydrophobic substrates such as hydrocarbons is an adaptation at the physiological and molecular level. During growth on hydrocarbons, ultrastructural changes such as cytoplasmic modifications [15], invagination of cytoplasmic membrane, and formation of inclusion bodies [6], fimbriae [30] and vesicles [14,31] have been reported. Adaptive mechanisms to aliphatic hydrocarbons differ between bacterial species, so that models do not hold good for all bacteria [36]. It is therefore not surprising that cells growing on hydrocarbons and using them as carbon and energy source have developed different ways of surviving in contact with hydrocarbon layers.

Hydrocarbon-degrading bacteria may be involved in indirect interaction between cell surface and hydrocarbon. In this case solvent transport is mediated by production of different types of emulsifying molecules to dissolve the hydrocarbons in water [7,10], thus avoiding direct contact between membrane phospholipids and the solvent layer. Direct contact between hydrocarbons and cells may also occur [11]. We recently investigated the mechanisms that allow *A. venetianus* VE-C3 to grow on diesel fuel [5]. It had already been observed that hydrocarbon-induced cells of this strain form a capsular polysaccharide (CPS) and biofilm on diesel oil droplets. The VE-C3 strain probably has an alternative emulsification mechanism for aliphatic hydrocarbons. We pro-

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posed incorporation of alkane nanodroplets in the CPS as a more realistic mechanism for the continuing growth of VE-C3. A previous experiment [5] clearly demonstrated that VE-C3 cells with CPS establish molecular contact with the hydrophobic surface of mercury drops of electrochemical probes (polarography technique). It was therefore postulated that a three-dimensional network of the CPS hydrogel entangles nanosized fuel droplets, which do not coalesce, and stabilizes the emulsion without reducing interface tension. Formation of a composite material is not a common concept in bacterial interaction with substrates but a well known phenomenon in materials science. The physical contact of two materials, hydrocarbons and CPS at the nanolevel, confers new properties on the mixture without formation of chemical bonds. In order to observe the fine structure of this composite material and cell clusters on diesel fuel, a new approach is suggested: the aggregates are studied by means of lectins (proteins specific for sugar residues), using transmission electron microscopy (TEM) to define subcellular details.

2. Materials and methods

2.1. Preparation of hydrocarbon-induced and uninduced cells

Acinetobacter venetianus strain VE-C3 was isolated from Venice Lagoon (Italy) [4] and underwent molecular characterization [8]. RAG-1 was used as a control strain because it belongs to the same species, *A. venetianus* [35], but it produces fimbriae [30] and the emulsifying agent, emulsan [10]. Both strains were stored at –80 °C and resuscitated by incubation at 28 °C under shaking in PCA medium, consisting of 5 g tryptone, 2.5 g yeast extract and 24 g NaCl per liter of deionized water. To obtain hydrocarbon-induced cells, the strain was grown in 10 ml of mineral medium, containing (per liter of distilled water): 24 g NaCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.7 g KCl, 2 g KH_2PO_4, 3 g Na_2HPO_4, 1 g NH_4NO_3 [22] and diesel fuel, consisting of a mixture of *n*-alkanes (C_{12}–C_{28}) as shown by gas chromatography analysis [4]. The solvent was filtered with 0.2 µm pore diameter teflon filters (Millipore) and added at a concentration of 2.0% (v/v) as sole carbon and energy source. Hereafter this medium is referred to as MMH (mineral medium with hydrocarbons). Hydrocarbon-induced cells lost their ability to grow immediately on hydrocarbons after five sequential transfers to PCA medium, and a lag-phase of 22 h was observed when cells were again transferred to MMH, as reported in other studies of this strain [4,5,8,11].

2.2. Microscope observations of cell glycosylation

Specimens were analyzed by transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM) and SDS polyacrylamide gel electrophoresis using ConA lectin from *Canavalia ensiformis* and PNA lectin from *Arachis hypogaea*, conjugated with biotin for blotting, ultradense ferritin for TEM and fluorescein isothiocyanate (FITC) for CLSM analyses. ConA and PNA lectins are widely used to study glycosylation in eukaryotic cells [21,23]. ConA is considered to be a good marker of *N*-linked glycans since it strongly binds their trimannosyl core [2]. PNA binds the Galβ−1,3GalNAC sequence in general, and sometimes certain *O*-linked glycans [28]. Although little is known about the carbohydrate determinants recognized in prokaryotes [21,23], the lectins used in this study turned out to be useful for identifying biochemical changes in glycoconjugate composition of the outer cell layers of VE-C3 in response to adaptation to diesel fuel.

To avoid artefacts and to perform immunolabeling tests directly on slides, all specimens were prepared for TEM by the freeze substitution method, fixing cell structures at –140 °C in liquid propane. The cells were gently dehydrated by replacing water with acetone and finally solidified with LR resin [19]. This type of resin inclusion is generally used for observation of eukaryotic cells by immunoblotting, because it enables specimens to be stained in situ, directly on thin sections, with lectins or other specific molecular markers (antibodies) conjugated with ultradense colloidal gold or ferritin, as in this study. Cells of VE-C3 and RAG-1 were grown in MMH and PCA media. After 48 h of incubation at 28 °C, cells of the two strains were harvested separately by centrifuging at 11 000 *g* and then frozen by immersing in liquid propane for 30 s. Frozen cells were immersed in acetone to displace water during the transition from the low temperature of liquid propane to room temperature [17,18]. Samples were then embedded in white LR resin [19]. Ultrathin sections were prepared using a LKB II Ultratome. Sections 0.02 µm thick were cut for TEM in alternating sequence with 0.80 µm thick sections for CLSM in order to analyse the same specimens with both techniques. Sections were then ready to be incubated for 1 h with 0.2 or 0.02 mg ml^{-1} ConA or PNA lectins, both conjugated with fluorescein isothiocyanate (FITC) for CLSM analysis or with ultradense ferritin for TEM analysis. Specimens were fixed on suitable supports (metal grids), washed and rinsed in PBS buffer (pH 7.0) containing 1.55 g K_2HPO_4 and 0.85 g $NaH_2PO_4 \cdot H_2O$ per liter of distilled water. As control, a second slice of each strain was incubated for 30 min in PBS buffer containing 0.1 M glucose or galactose, and then incubated with ConA or PNA at the same concentrations as for the experiments reported above in the presence of the corresponding hapten in order to reveal non-specific binding of the lectins to non-haptenic structures [9].

Fluorescence was imaged by CLSM (mod. MRC-500; Biorad Microscience Division). Three-dimensional images were obtained by a procedure already reported [3]. TEM observations were performed with a JEM 100b electron microscope (JEOL, Tokyo, Japan). Some images were taken without counterstaining to enhance the effects of glycosylation on cells. To show that there is some specificity of the lectin stain, specimens were also counterstained with 3.0% uranyl

acetate solution for 15 min, washed once with distilled water and incubated in lead citrate for 10 min. Although image definition was lower than with the conventional method, this technique enabled reactions to be carried out directly on slides.

2.3. Calculation of relative fluorescent intensity (RFI)

RFI determined as pixels per unit surface area (cm^2) is related to the concentration of specific binding of lectins ConA and PNA conjugated with FITC to sugar residues [9]. RFI was measured by SCLM, maintaining optical and computer parameters constant. Original photos of specimens were exported in Tiff format to a Power Macintosh G3 and measured on 18×15 cm resized photos using Photoshop 4.0 to normalize all measurements. Sampling areas of 1 cm^2 were taken randomly five times in the areas of fluorescence. Pixel cm^{-2} concentrations were calculated and standard deviations measured by NIH image 1.62b software. These experiments were also performed with the RAG-1 strain with hydrocarbon-induced and uninduced cells in MMH and PCA media as control cultures.

2.4. Lectin-blotting

Cells of VE-C3 and RAG-1 strains were grown in PCA medium and MMH for 24 h at 28 °C, harvested by centrifuging at 6000 *g* for 20 min and washed twice with PBS. Cells were resuspended in 2 ml 10 mM Tris–HCl (pH 8.1) and disrupted on melting ice with a sonicator (Labsonic U Braun), duty cycle 30%, for 10 min. Membranes were purified substantially as described by Myers and Myers [25]. Disrupted cells were incubated for 10 min on ice with 2 µl 1 M $MgCl_2$ and a few crystals of DNase I and RNase A. One-tenth volume of lysozyme (6.4 mg ml^{-1}) was added and incubated for another 20 min. After centrifuging at 1450 *g* for 20 min, cell debris was removed and cell extracts were centrifuged for 150 min at 177 500 *g* in a Beckmann type 70 Ti rotor. Pellets were resuspended in 0.5 ml 10 mM HEPES (pH 7.5) and dialyzed extensively against the same buffer [18]. Protein concentration was determined by BCA protein assay (Pierce Chem. Co., Rockford, IL, USA).

Equivalent amounts of protein were separated from both strains by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) [16] using a 16% acrylamide separation gel. The proteins were stained with Coomassie blue or transferred overnight to nitrocellulose [34]. The nitrocellulose sheets were blocked with 0.2% non-fat powdered milk in TBS (20 mM Tris–HCl, 500 mM NaCl, pH 7.5) and incubated at room temperature with ConA or PNA lectins conjugated with biotin for 1 h at final concentrations of 10 µg ml^{-1} and 25 µg ml^{-1}, respectively. After several washings with TBS containing 0.1% Tween-20, blots were incubated for 1 h with extravidin conjugated with alkaline phosphatase (BioRad, Cambridge, MA, USA) at a dilution of 1:1000. After extensive rinsing in TBS-Tween, labeled proteins were developed using the Immun-star chemiluminescent protein detection system (Biorad Microscience, Cambridge, MA) according to the manufacturer's instructions. Lectin incubation was omitted, as control, to reveal non-specific staining due to extravidin binding.

3. Results

Our previous study suggested that the increasing hydrophobicity of VE-C3 measured by the MATH test is most likely induced by incorporation of diesel fuel droplets into the CPS matrix, facilitating mass transfer of hydrocarbons from the medium to the cells. To observe this composite material, the following experiments were performed.

3.1. Envelope glycosylation

A dense distribution of glucose/mannose target molecules was observed in situ by CLSM with fluorescein conjugated to ConA lectin on the cell surface of hydrocarbon-induced VE-C3 cells (Fig. 1A). The specimen of VE-C3 growing in MMH, preincubated with glucose and then stained with ConA lectin in the presence of the competing hapten (control), did not show any lectin binding to structures on the outer cell membrane (Fig 1B). This control experiment demonstrated that the fluorescence was due to specific binding of the lectin to glucose and/or mannose containing molecules of the cell membrane (Fig. 1A). The same test was performed with PNA lectin (Fig. 1C) and the control was incubated with the respective hapten galactose (Fig. 1D). The fluorescence emitted was much less than with ConA. Further control experiments, also using a strain of *Acinetobacter* RAG-1 analyzed by the same techniques, were performed to determine the relative emission of fluorescence in different controls.

Fluorescence was measured as relative intensity cm^{-2} in photos of different specimens and media by CLSM image analysis (Fig. 2). The hydrocarbon-induced VE-C3 strain stained with ConA-FITC showed the highest fluorescence (159 ± 6.8 pixel cm^{-2}). Control cells preincubated with the hapten glucose had significantly lower fluorescence (28 ± 0.9 pixel cm^{-2}). Hydrocarbon-induced RAG-1 strain stained with ConA also had low fluorescence (44.1 ± 6.2 pixel cm^{-2}). Fluorescence emitted by PNA bound to cell walls was less intense than for ConA (Fig. 3). The relative intensity of strain RAG-1 (60.3 ± 9.9 pixel cm^{-2}) was similar to that of strain VE-C3 (51.6 ± 9.5 pixel cm^{-2}), and higher than in the control preincubated with its hapten, galactose (28.3 ± 1.4 pixel cm^{-2}). The emissions of ConA and PNA in both uninduced strains, cultivated in PCA medium without diesel fuel, were even lower.

Uninduced VE-C3 cells grown on PCA medium (without hydrocarbons) were free-living coccoid cells, surrounded by an outer membrane with even fibril distribution (arrow) (Fig. 3A), whereas in hydrocarbon-induced bacterial cells

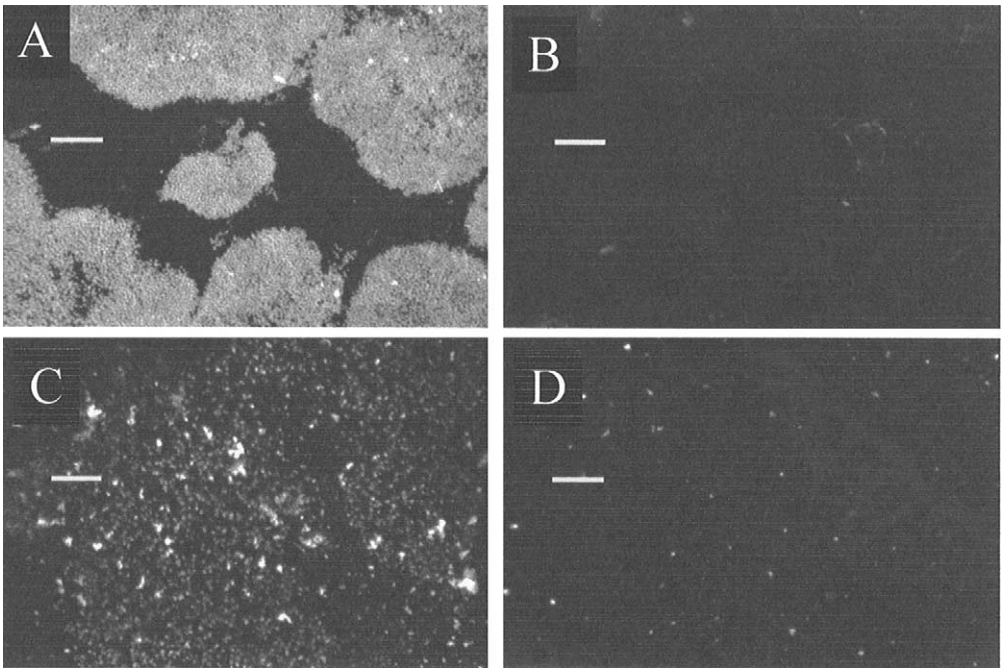

Fig. 1. CLSM photos of *A. venetianus* VE-C3 aggregates in MMH. Bars are 12.5 µm. The differences between specimens treated and not treated with the hapten of the lectin show the distribution of glycosylated structures producing a biofilm on diesel fuel without aspecific staining. (A) Fluorescence due to binding of lectin ConA-FITC to glucose and/or mannose residues on cell aggregates. (B) The same specimen pretreated with the hapten glucose to determine the specificity of this lectin for sugar targets (control). (C) Fluorescence due to binding of lectin PNA-biotin to galactose residues with avidin-FITC counterstain on cell aggregates. (D) The same specimen pretreated with the hapten galactose to determine the specificity of this lectin towards its sugar target (control).

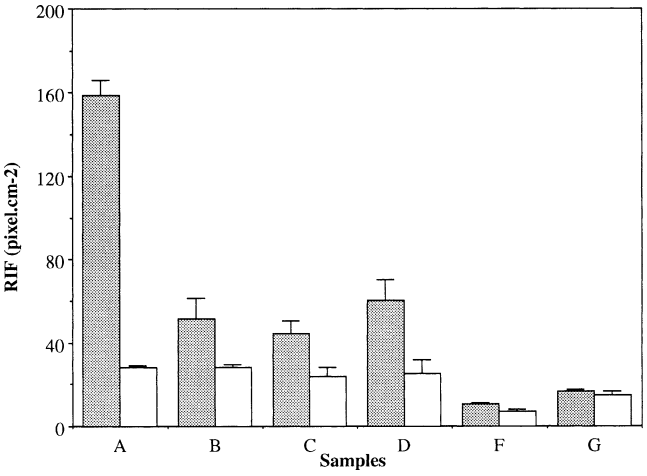

Fig. 2. RFI in pixel $cm^{-2} \pm$ standard deviation determined from CLSM photos resized (18 × 15 cm) on the computer screen. (A) RFI (see Fig. 2A) of ConA lectin bound to VE-C3 grown on MMH (gray column) and its control (see Fig. 2B) pretreated with the hapten glucose (white column). (B) RFI of PNA lectin (Fig. 2C) bound to VE-C3 strain grown on MMH medium (gray column) and its control (see Fig. 2D) pretreated with the hapten galactose (white column). (C) RFI of ConA lectin bound to RAG-1 cells grown in MMH medium (gray column) and its control pretreated with the hapten glucose (white column). (D) RFI of PNA lectin bound to RAG-1 strain grown in MMH (gray column) and its control pretreated with the hapten galactose (white column). (F) RFI of ConA lectin bound to VE-C3 strain grown in PCA (gray column) and its control pretreated with the hapten glucose (white column). (G) RFI of PNA lectin bound to VE-C3 strain grown in PCA (gray column) and its control pretreated with the hapten galactose (white column).

grown in MMH, fibrils were concentrated and linked in bundles, showing few cell-to-cell contact points. Changes in cell morphology ranging from coccoid to deformed shapes were also observed (Fig. 3B).

When microscopy sections of cells were stained with ConA-ferritin (immunoblot-like technique), without counterstaining, the ConA-ferritin localized in the CPS, a thick glycosylated layer around the cell walls (Fig. 3C). When cells were stained with PNA-ferritin, fibrils containing galactose were observed connecting adjacent cells (Fig. 3D).

To better define the detail of these glycosylated structures at the subcellular level, the ConA-ferritin (0.02 mg ml^{-1}) labelled cells were counterstained with uranyl acetate to enhance cell definition and the specificity of the lectin stain. This further staining brought out the ultrastructure of connecting cells (Fig. 4A) and diesel fuel nanodroplets, which were embedded in the polysaccharide matrix. This is the image of the mixed hydrocarbon-polysaccharide (composite material). The physical inclusion of diesel fuel in polysaccharides is certainly responsible for the significant hydrophobicity increases in VE-C3 cells already reported [5]. To determine differences due only to glycosylation, the control specimen was pretreated with hapten glucose. CPS and the composite material were less evident, whereas a peripheral belt of diesel fuel nanodroplets, previously covered by CPS structure, became more evident at the envelope perimeter (Fig. 4B)

When specimens were stained with PNA-ferritin (0.2 mg ml^{-1}) and then counterstained with uranyl acetate,

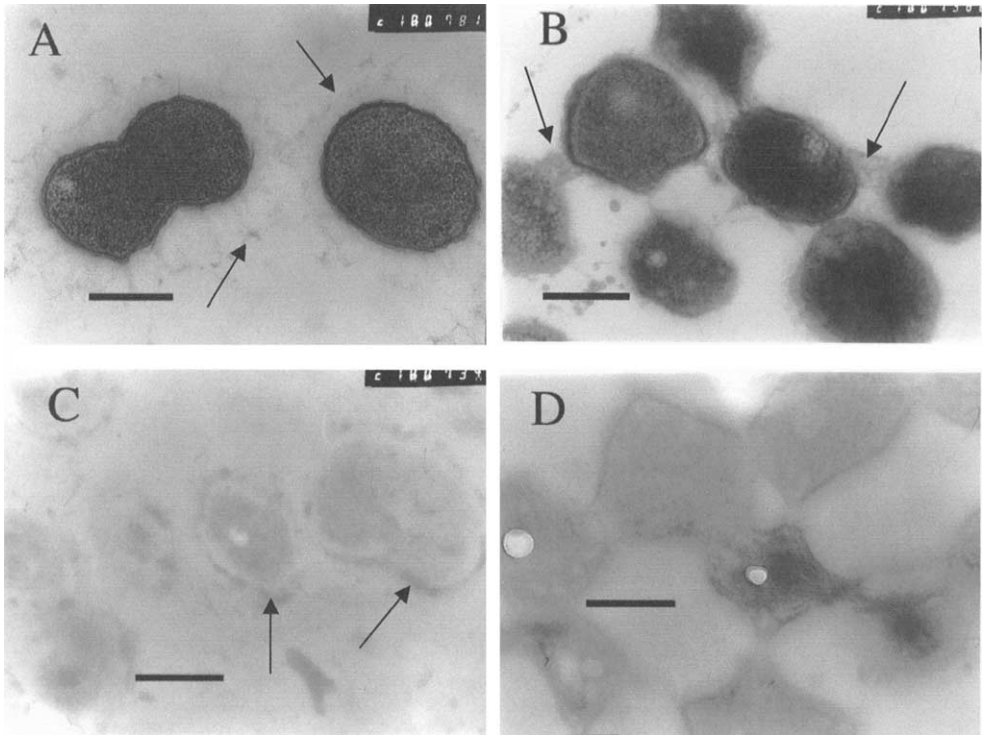

Fig. 3. TEM photos of *A. venetianus* VE-C3 in PCA and MMH. Bars are 0.5 µm. (A) Uninduced free-living turgid coccoid cells, grown in PCA medium and stained with uranyl acetate. Note peripheral fibrils. (B) Hydrocarbon-induced cells of VE-C3 grown in MMH and stained with uranyl acetate. Note dense network of fibrils between deformed aggregating cells. (C) Hydrocarbon-induced VE-C3 grown in MMH and stained with ConA-ferritin without counterstaining with uranyl acetate. Note thickening of walls (arrows) due to capsular polysaccharide formation. (D) Hydrocarbon-induced VE-C3 grown in MMH and stained with PNA-ferritin without uranyl acetate counterstaining. Note fibrils (arrows) responsible for cell aggregation.

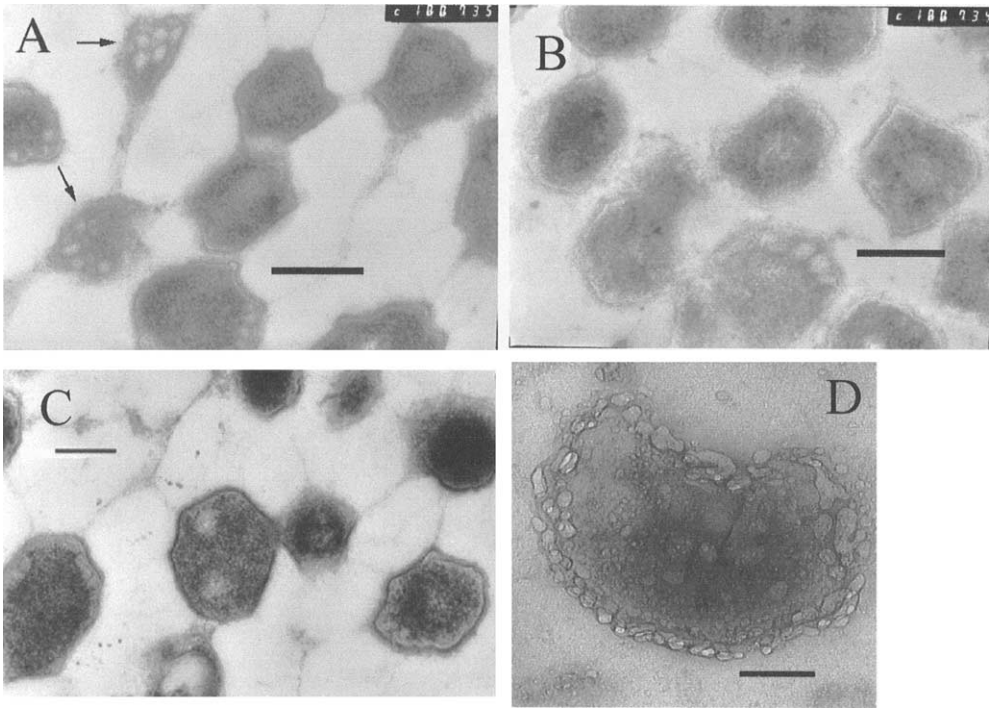

Fig. 4. TEM photos of hydrocarbon-induced *A. venetianus* VE-C3 grown in MMH and stained with 0.02 mg ml^{-1} ConA-ferritin (A, B) and PNA-ferritin (C, D) and counterstained with uranyl acetate. Bars are 0.5 µm in A, B, C. (A) Cells connected by dense fibrils; arrows indicate composite material containing nanodroplets of diesel fuel. (B) The same specimens pretreated with the hapten glucose as control and then stained with ConA-ferritin. Note absence of cell connections and composite material. (C) Cells showing dense PNA-positive fibril network between cells. (D) Detail of same specimen pretreated with its hapten galactose as control and stained with PNA-ferritin. Note diesel fuel nanodroplet belt around cell envelope. Bar is 0.15 µm.

dense fibril bundles connecting hydrocarbon-induced cells were indicated by galactose residues (Fig. 4C). In control specimens pretreated with the hapten galactose, the fibrils disappeared (Fig. 4B) and merged backward the fibril-naked cell, a dense peripheral belt of evenly distributed diesel-fuel nanodroplets (Fig. 4D). Cell details showed intense emulsification of diesel fuel producing a nanodroplet belt averaging 20–80 nm at the phospholipid membranes. This suggests that during capsule production, other molecules, such as an outer membrane protein, may also be glycosylated.

3.2. Glycoproteins

Glycoproteins embedded in outer membranes have emulsifying activity, as previously observed in another hydrocarbon-degrading species, *Acinetobacter radioresistens* KA53 [26,33]. In studying glycoproteins of VE-C3 strain to explain the distribution of diesel fuel nanodroplets at the cell envelope, we observed a large reduction in oil surface tension. Outer membrane protein profiles (Fig. 5) of hydrocarbon-induced cells (lane A) and uninduced cells (lane B) showed that the number of outer membrane proteins in VE-C3 cells grown in PCA medium was probably higher than that of cells grown in MMH. The number of PNA-positive bands decreased in hydrocarbon-induced cells grown in MMH. Bands stained with PNA-biotin consisted mainly of a 18-kDa glycoprotein accompanied by minor bands with higher molecular mass above 33.4 kDa. Several outer membrane proteins were revealed by PNA lectin-blotting analysis from extracts of uninduced cells.

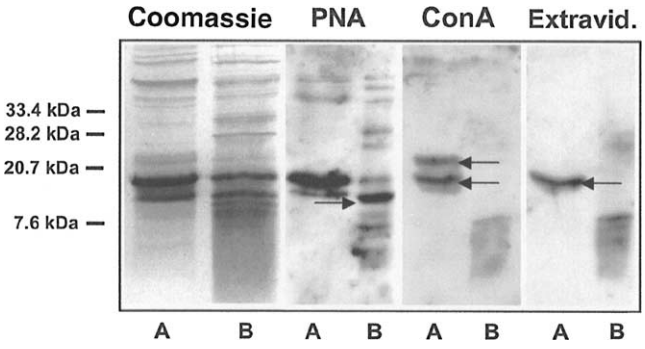

Fig. 5. Polyacrylamide gel electrophoresis (10 μg total protein per lane) and lectin-blot of outer membrane extracts of hydrocarbon-induced VE-C3 cells grown on MMH (lane A) and uninduced cells grown on PCA medium (lane B). Extracts of induced VE-C3 grown on MMH and uninduced cells in PCA after staining with PNA-biotin and then with extravidin conjugated with alkaline phosphatase revealed a strong band of PNA-positive glycoprotein (arrow), also present in uninduced cell extracts. The same samples treated ConA-positive glycoproteins were not present in uninduced cells (lane B) grown on PCA medium. Whereas in hydrocarbon-induced VE-C3 cells grown on MMH, treated with ConA, revealed two ConA-positive glycoproteins (arrows): a specific one with an apparent molecular mass of 22 Kda (upper arrow) and a non-specific one (lower arrow). The latter was probed with extravidin alone (control), in the next to last lanes, where a prominent streptavidin-binding protein was found corresponding to the ConA-positive band of lower molecular weight (arrow).

Hydrocarbon-induced cells grown in MMH showed two ConA-positive glycoproteins migrating with an apparent molecular mass of 18 and 22 kDa, respectively. Membrane proteins from uninduced cells did not react with ConA staining, whereas extravidin staining without lectins (control) revealed a protein band at 18 kDa in the outer membrane of hydrocarbon-induced cells. This avidin-binding protein was not glycosylated, but suggested the presence of biotin.

4. Discussion

An objective of this study was to describe the fine structure of composite material formed by *A. venetianus* VE-C3 in the presence of diesel fuel. The study was performed with a new approach for prokaryotic cells. Lectins were used to recognize sugar residues, in the same way that antibodies and conjugates with ultradense colloidal gold or ferritin are used to recognize protein epitopes in immunoblotting. Specific sugar recognition was performed directly on microscope thin sections. For TEM and CLSM observations, this was done by freeze substitution, embedding specimens in a special resin suitable for in situ biochemical treatment. Specimens were treated with lectins with or without the respective haptens, namely glucose/mannose in the case of ConA lectin, and galactose for PNA lectin. This direct approach enabled us to avoid measurement of total sugars in cultures by means of non-specific agents, such as the proteases, glycosidases and surfactants customary in cell adhesion studies [1,27].

The presence and distribution of ConA- and PNA-positive sugar-residue determinants were studied in cell aggregates (CLSM), subcellular structures (TEM) and molecular constituents (PAGE) of *A. venetianus* VE-C3 outer membrane. The distribution of ConA-positive glycosylation around the cell wall (CSLM and TEM) indicated CPS induced by diesel fuel. CLSM analysis gave information about relative concentrations of ConA, determined as RFI by image analysis, and the role of CPS in cell adhesion to diesel fuel to form aggregates of hundreds of thousands of cells. TEM revealed details of CPS constituents consisting of composite material and fibrils. Lectin blotting (PAGE) revealed the location and molecular weight of ConA and PNA glycoproteins in the outer membrane.

PNA glycoproteins are involved in adhesion processes, whereas ConA glycoprotein protects VE-C3 cell envelope phospholipids from direct contact with *n*-alkanes. The protective role of polysaccharide has been observed in other encapsulated cells of *Acinetobacter* sp. [29]. Intense relative fluorescence due to binding of ConA lectin-FITC to glucose/mannose residues in diesel fuel-induced cells of *A. venetianus* VE-C3 indicates thick glycosylation of the outer membrane, which was not observed in RAG-1.

The composite material of interest, a mixture of free CPS and diesel fuel, was observed directly by TEM [10] (Fig. 4A). Diesel fuel nanodroplets were visibly embed-

ded as vesicles in a ConA-positive polysaccharide matrix. This physical structure had higher viscosity than the separated components, which were in contact without reacting. This hydrocarbon incorporation mechanism by polysaccharides is supported by recent studies on giant polysaccharide aggregates that periodically bloom in the northern Adriatic [13,20]. Moreover, *n*-hexadecane could be incorporated into this hydrophilic gel to form a hydrophobic material of higher viscosity than the original gel. A gel prepared from dextran (MW 50×10^6) in seawater had a similar capacity to incorporate *n*-hexadecane droplets and form a material of higher viscosity. These vesicles of composite material raise hydrophobicity and incorporate nanodroplets in cell wall. VE-C3 cells in seawater medium were found to be hydrophilic with zeta potential $\zeta = -10.5 \pm 0.35$ mV in medium without diesel fuel, whereas RAG-1 cells were less hydrophilic ($\zeta = -4.9 \pm 1.42$ mV) [5]. Hydrocarbon incorporation and vesicle formation in cells is a known process [5,12,14], whereas vesicles composed of hydrocarbons and polysaccharides are a new finding. Other molecular constituents cannot yet be excluded.

The diesel fuel nanodroplets distributed around VE-C3 cell envelopes measured 10–60 nm. This fragmentation of diesel fuel at the cell wall suggests strong bioemulsifying activity. *A. venetianus* VE-C3 has hemolytic activity when cultivated on agar-blood medium. This phenotypic character is generally used as a marker for quick screening of oil-degrading bacteria with emulsifying activity [24]. The finding of diesel fuel-inducible 22 kDa ConA-positive glycoprotein in VE-C3 cells therefore suggests that this glycoprotein may have an emulsifying role (not yet tested). It was recently found that a glycoprotein in *A. radioresistens* KA53 plays an emulsifying role [32,33]. The bioemulsifier of the KA53 strain, referred to as alasan [26], is a high molecular weight complex of polysaccharide and protein. An alasan protein with an apparent molecular mass of 45 kDa, almost double that of our ConA-positive glycoprotein, was recently purified and shown to account for most of the emulsifying activity. The *N*-terminal sequence of the 45-kDa protein showed high homology with an OmpA-like protein from *Acinetobacter* spp. [33].

The other PNA-positive glycoproteins are constitutive and mainly involved in fibril networking between cells to form a continuous monolayer biofilm, as already demonstrated by polarography [5]. PNA-positive hydrophilic fibrils are randomly distributed around the cells in PCA medium (Fig. 3A), whereas the PNA-positive polysaccharide fibrils of VE-C3 tend to coalesce to form bundles of fibrils in contact with the hydrophobic surface of diesel fuel (Fig. 3B). These hydrophilic contacts (sugars) on a hydrophobic layer (diesel fuel) are based on attraction and repulsion of hydrophilic and hydrophobic forces and their modification due to molecular rotations, typical of polysaccharides. Disordered structures of polysaccharides in aqueous medium tend to order at the oil/seawater interface due to high ionic strength ("salt jump"). At the moment, this seems the most probable mechanism of microbial biofilm formation on droplets of fuel oil.

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