

Characterization of exopolymeric substances (EPS) produced by *Aeromonas hydrophila* under reducing conditions

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The aim of this work was to investigate the production of extracellular polymeric substances (EPS) by *Aeromonas hydrophila* grown under anaerobic conditions. EPS composition was studied for planktonic cells, cells attached to carbon fibre supports using a soluble ferric iron source and cells grown with a solid ferric iron mineral (gossan). Conventional spectrophotometric methods, Fourier transform infrared (FTIR) and confocal laser scanning microscopy (CLSM) were used to determine the main components in the biofilm extracted from the cultures. The key EPS components were proteins, indicating their importance for electron transfer reactions. Carbohydrates were observed mostly on the mineral and contained terminal mannosyl and/or terminal glucose, fucose and *N*-acetylgalactosamine residues.

Keywords: *Aeromonas hydrophila*; extracellular polymeric substances; CLSM; FTIR; SEM

Introduction

Microorganisms grow in natural environments usually in the form of interfacial films bound to different substrata. Bacterial biofilms are composed of a hydrated matrix of extracellular polymeric substances (EPS) which form their immediate environment. The typical constituents of EPS are polysaccharides, proteins, nucleic acids and lipids (Flemming et al. 2007). These compounds provide the mechanical stability for these biofilms (Sutherland 2001), mediate their adhesion to surfaces (Lüttge et al. 2005) and form a cohesive three-dimensional polymer network interconnecting and immobilizing cells. EPS can be subdivided into bound/capsular EPS and soluble/colloidal EPS (Sheng et al. 2010). Capsular EPS are tightly bound to cells, while colloidal EPS are weakly bound to cells or easily lost into the solution. Generally, these two types of EPS can be separated by centrifugation, with those remaining in the supernatant being soluble, colloidal EPS and those forming microbial pellets being bound capsular EPS. EPS of the biofilm matrix appear to have several key properties and functions. Polymers represent a structural component of microbial cell surfaces. EPS may carry charged or hydrophobic groups resulting in an adsorptive polymer which can bind various nutrients, metals and contaminants (Harrison et al. 2007). Some EPS are involved in informative functions (Whitchurch et al. 2002), whereas others are able to condition interfaces (Zhong et al. 2007). Moreover, some polymers represent an ideal source of nutrients (Davey & O'Toole 2000) and some can be

involved in motility (Lu et al. 2005). Although speculative, some evidence shows that there is a potential role for redox-active EPS used by certain bacteria for anaerobic respiration (Neu & Lawrence 2009). Extensive research undertaken in the past few decades has focused on understanding the properties of these biopolymers. Several analytical techniques are used for studying EPS components. The use of Fourier transform infrared spectroscopy (FTIR) in the field of microbiology has proved to be a promising technique. FTIR spectroscopy simultaneously measures the vibrations of functional groups of different cell components in multi-component mixtures (Schmitt & Flemming 1998; Sheng et al. 2006). Furthermore, there has been the development and application of techniques allowing the observation of fully hydrated interfacial microbial cultures using confocal laser scanning microscopy (CLSM) (Neu & Lawrence 1997; Neu et al. 2001; Zippel & Neu 2011).

In anaerobic soil and sedimentary environments, microbial reductive dissolution of iron oxides coupled to oxidation of organic matter (electron donor) is thought to be an important biogeochemical process, resulting in the formation of biogenic ferrous iron (Fe(II)) species.

Gossan ores are natural minerals composed mainly of iron oxyhydroxides with residual unaltered minerals such as quartz. In addition to iron oxyhydroxides, various precious metals, such as gold and silver, and various sulphate and silicate minerals can occur in gossans (Essalhi et al. 2011). Anaerobic iron bioleaching is ubiquitous in

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subsurface environments, including mining-impacted areas. The extent of iron oxide microbial dissolution in subsurface sediments can vary greatly depending on different environmental conditions (Bonneville et al. 2009; Salas et al. 2010) including the specific bacteria involved in this process, the surface area, particle size and crystallinity of iron oxides, the availability of electron donors, and the interaction between iron oxides and bacteria.

The immobilization technology of cells and enzymes has rapidly developed and has many applications. Cell immobilization is characterized by maintaining a stable high density of cells on the support matrix and could improve the reductive rate of Fe(III) reduction. It has been applied to treat acid mine-drainage or bioleach minerals. Another application of cell immobilization could be bacterial fuel cells. Some species can colonize the anode and directly transfer electrons to the electrode from the bacterial EPS that are in contact with it (Torres et al. 2010).

In the present study, the colloidal and capsular EPS fractions obtained from pure cultures of *Aeromonas hydrophila* are characterized. The aim was to identify the constituents of EPS by chemical extraction using EDTA and their spatial interactions with each other and with the support. This work was focused on the EPS of the Gram-negative bacterium *A. hydrophila*, which is known to reduce Fe(III) and keep Fe(II) in solution (García-Balboa et al. 2010). For that purpose, *A. hydrophila* was cultivated using soluble ferric citrate as iron source. The EPS were extracted from planktonic cells and from cells grown on carbon fibres. Moreover, the EPS produced by microorganisms grown on a gossan ore as an insoluble iron source were studied. The formation of *A. hydrophila* biofilms on different substrata under iron reducing conditions was examined using scanning electron microscopy (SEM) and CLSM. Fluorescently labelled lectins were used to stain the EPS, in order to gain hints about the chemical composition of the polysaccharides in the biofilm.

Materials and methods

Bacterial culture

The *A. hydrophila* strain for the study was isolated from the edge of an open-pit lake surrounding an abandoned mine site named 'Brunita' (formerly a source of Pb–Zn ores) near La Unión (Murcia, Spain).

A modified Postgate C medium was used to grow the bacterium. It contained the following salts (g l^{-1}): KH_2PO_4 , 0.5; NH_4Cl , 1.0; Na_2SO_4 , 4.5; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.06; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06; sodium lactate, 6.0; yeast extract, 1.0; and sodium citrate heptahydrate, 0.3. The pH was adjusted to 7.0 ± 0.2 .

Standard anaerobic techniques were used in this research. Sterilized medium (autoclave conditions: 121°C , 30 min) was poured into individual glass flasks. The medium was supplemented with ferric citrate

(60 mM in Fe^{3+}) or with the gossan mineral (10 g l^{-1}). Then, the solution was vigorously bubbled with $\text{N}_2:\text{CO}_2$ (80/20, v/v) to strip dissolved oxygen. The flasks were capped with butyl rubber stoppers and sealed with aluminium crimps. A 10% inoculum of a Fe(III)-reducing culture was used ($8 \times 10^8 \text{ cells ml}^{-1}$). The cultures were incubated unstirred at 30°C in darkness.

EPS extraction method

The extraction protocol is summarized in Figure 1. When the cultures reached the stationary growth phase, they were centrifuged for 10 min at 7,500 rpm (9,900 g) and 4°C . The supernatant was collected and filtered through $0.2 \mu\text{m}$ pore size filters under sterile conditions to eliminate remaining bacteria. The fraction obtained contained the colloidal EPS. To extract the bound EPS, the pellet was re-suspended in 10 ml of salt solution and centrifuged for 10 min at 7,500 rpm (9,900 g) and 4°C . The supernatant was named the washed fraction. The new pellet was re-suspended in 10 ml of 20 mM EDTA at pH 7 and the suspension was incubated with shaking for 1 h at 4°C . Thereafter, the mixtures of bacterial suspensions and extracting agents were centrifuged for 10 min at 7,500 rpm (9,900 g) and 4°C to remove the remaining cells. This process was repeated three times and the three resulting supernatants contained the capsular fraction. To remove the residual salts from the growth medium and the extraction agent, each EPS fraction was dialysed, first with deionized water for 12 h and then with milliQ water for 72 h at 4°C . Cellulose membrane dialysis tubing of 3,500 Da was used.

The EPS mass was obtained after freeze drying 2 ml of the purified EPS solution. The freeze drying was performed using a freeze dryer ALPHA 2-4 LSC at 1 mbar and -80°C for 24 h (Christ, Osterode am Harz, Germany).

Characterization of the chemical composition of the EPS

Carbohydrates, proteins, uronic acids and DNA were determined photocolometrically using a UV–Vis spectrophotometer (JASCO V-650, Jasco, Tokyo, Japan). The amount of carbohydrate was determined by the Dubois assay (Dubois et al. 1956) using glucose as standard and a wavelength at 490 nm. The protein content was determined according to the Lowry method (Lowry et al. 1951) using bovine serum albumin (BSA) as standard and measuring absorbance at 595 nm. The quantitative determination of uronic acids was performed using the method of Blumenkrantz and Asboe-Hansen (1973). D (+)-glucuronic acid was used as standard and the absorbance was measured at 520 nm. The extent of EPS contamination by cell lysis during extraction was estimated by analysing the quantity of 2-keto-3-deoxyoctonate

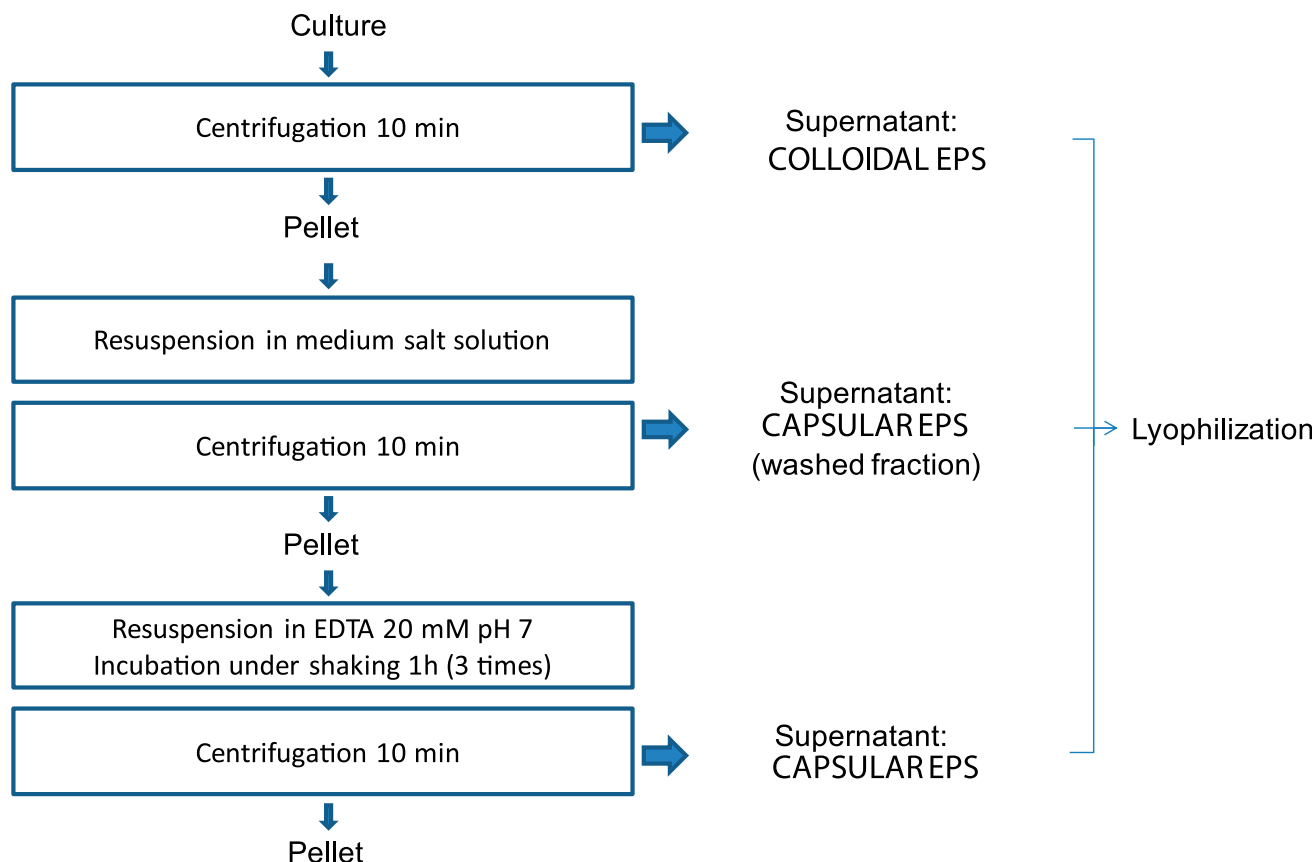


Figure 1. General procedure for EPS extraction using EDTA.

(KDO) in samples using KDO as a standard (Karkhanis et al. 1978).

Fourier transformed infrared (FTIR) spectroscopy

FTIR was used to determine the variation in the chemical groups in the EPS due to different growth conditions. Previous to FTIR analysis, the EPS sample and the control were freeze-dried (ALPHA 2-4 LSC, -80°C). These samples were pelleted, mixing the EPS sample with ~ 250 mg of KBr. The spectra were recorded with a Nicolet Magna 750 in the region of $500\text{--}4,000\text{ cm}^{-1}$ at a resolution of 4 cm^{-1} (Thermo Scientific, West Palm Beach, FL, USA).

Scanning electron microscopy (SEM)

Cultures were grown under different conditions and filtered onto a $0.2\text{ }\mu\text{m}$ pore-size filter. The samples were successively dehydrated with acetone and stored overnight at 4°C in 90% acetone. The samples were dried by critical-point drying and coated with graphite and gold. Specimens were examined with a JEOL JSM-6330F microscope, FE-SEM at 10 kV to determine the spatial

distribution of cells on the surfaces (JEOL, Tokyo, Japan).

Confocal laser scanning microscopy (CLSM)

The visualization of the fluorescent markers was performed using a laser scanning module LSM 510 coupled with an inverted microscope 100 M BP (Zeiss, Jena, Germany). The CLSM images were generated in a multi-track mode which allowed the separate recording of the different signals corresponding to the respective laser wavelength. Image analysis was performed with software programs LSM 510 version SP2 (Zeiss), Volocity 3.5 (Improvision) and AxioVersion 3.1 (Zeiss, Jena, Germany).

Nucleic acid staining

The spatial distribution of microbial cells within the aggregates was shown with general nucleic acid specific stains: DAPI (diamidino-2-phenylindole), SYTO 9 and SYTO 62. DAPI is a blue fluorescent probe that fluoresces brightly upon selectively binding to the minor groove of double stranded DNA. SYTO 9 is a green

fluorescent nucleic acid dye to stain live and dead bacteria. The cell-permeant SYTO 62, a fluorescent nucleic acid stain, exhibits bright and red fluorescence upon binding to nucleic acids.

Lectin binding assays

Ten lectins were selected to determine whether the EPS contained fucose, mannose, glucose, galactose, glucosamine, *N*-acetyl galactosamine or other residues. To label carbohydrates the following fluoresceine isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) labelled lectins were used in these experiments: *Arachis hypogaea* (PNA) specific for D(+)galactose, *Canavalia ensiformis* (Con A) specific for D(+)glucose and D(+)mannose, *Erythrina cristagalli* (ECA) specific for *N*-acetyl-D-galactosamine and D-galactose, *Ulex europaeus* (UEA I) specific for L(-)fucose, *Lens culinaris* (LcH) specific for α -mannose and α -glucose, *Triticum vulgare* (WGA) specific for *N*-acetyl-glucosamine, *Glycine max* (SBA) specific for α - and β -*N*-acetylgalactosamine and galactopyranosyl residues, *Phytolacca americana* (PWM) specific for *N*-acetyl-glucosamine, *Bandeiraea simplicifolia* (BS I) specific for α -D-galactosyl and *N*-acetyl- α -D-galactosaminyl residues and *Phaseolus vulgaris* (PHA-E) specific for galactose. Briefly, samples were incubated with 0.05 mg ml⁻¹ of the lectins for 40 min at room temperature. Stained samples were then washed three times with filter-sterilized tap water in order to remove the non-bound lectins. Direct light exposure was avoided as much as possible. In order to prolong the fluorescence of the dyes, an antifading agent (CitifluorTMAF2, Citifluor, Eschborn, Germany) was used when mounting the samples.

Results and discussion

EPS production and chemical composition

The EPS extraction yield using EDTA treatment has been considered to be relatively low compared with other chemical extraction methods for activated sludge (Zhang et al. 1999). For example, regular centrifugation with formaldehyde extraction results in a yield of 10%, whereas EDTA extraction reaches a yield of 2%. However, the EPS extraction method using EDTA allows detection of the protein content in the EPS. There is, therefore, a compromise between EPS yield and availability for specific analyses. In addition, no significant cell lysis occurs during EDTA extraction (Zhang et al. 1999), which makes this method a good choice for EPS extraction from biofilms. Cell lysis during extraction was estimated by analysing the quantity of 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) in the samples. KDO is part of the cell membrane of Gram-negative bacteria

and therefore can be used as a marker for contamination by membrane compounds. The amounts of KDO in the cell pellets after cell lysis with sodium dodecyl sulphate (SDS) were compared with that in EPS. KDO in the EPS fraction was detected after extraction for 9 h and was <5% of that in cell pellets. A low content of KDO indicated that there was no significant cell lysis causing intracellular materials to be released into the solution during the extraction.

Both colloidal and capsular EPS from *A. hydrophila* biofilms were extracted and analysed. Bacterial cells produced more capsular EPS, when forming biofilms, compared to planktonic cells (Figure 2). This evidence was also corroborated in previous studies on bacterial biofilm formation (Laspidou & Rittmann 2002; Cao et al. 2011). The increased amount of colloidal EPS when using a soluble iron source, citrate-Fe(III), was accounted for by the citrate remaining in this fraction after dialysis treatment. Citrate was not present when iron was provided as a solid mineral.

The composition of the EPS matrix in biofilms is reported to be very complex, containing proteins, carbohydrates, nucleic acids, lipids, amphiphilic molecules and humic substances (Flemming et al. 2007). In this study, conventional chemical colorimetric analyses were used to quantify the composition of the purified EPS produced by *A. hydrophila* and extracted from samples with different growth conditions. The results are listed in Table 1.

The EPS extracted from anaerobic cultures was mainly composed of proteins. However, the content of polysaccharides was very low. This fact may be

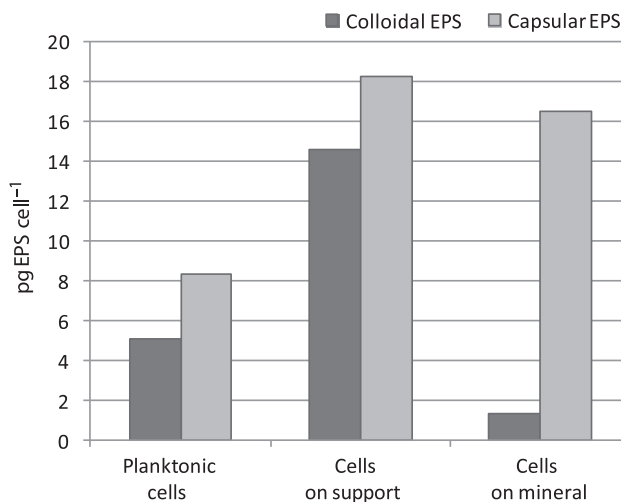


Figure 2. Amount of EPS produced per cell of *A. hydrophila* grown under different conditions and by planktonic cells, cells attached to carbon fibre supports using a soluble ferric iron source and by cells grown with a solid ferric iron mineral (gossan).

Table 1. Chemical composition of EPS produced by *A. hydrophila* grown under different conditions.

| | | % (wt/wt) of total EPS | | |
|------------------|-----------|------------------------|----------|--------------|
| | | Sugars | Proteins | Uronic acids |
| Planktonic cells | Colloidal | 0.18 | 1.48 | 1.04 |
| | Capsular | — | 22.9 | 3.49 |
| Carbon fibres | Colloidal | 0.51 | 2.03 | 0.92 |
| | Capsular | — | 27.04 | 2.37 |
| Gossan mineral | Colloidal | — | 7.52 | 0.94 |
| | Capsular | 0.43 | 37.77 | 1.75 |

explained by the transporter profile of *A. hydrophila* which is comparable to those of pseudomonads and vibrios, with an abundance of amino acid and peptide transporters and relatively few sugar uptake systems (Seshadri et al. 2006). *A. hydrophila* is able to reduce metals and proteins that could act as redox active molecules (Neu & Lawrence 2009), facilitating and directing electron transfer, and may aid in the efflux of heavy metals or toxic compounds encountered in potentially highly polluted water.

Polysaccharides were found in the capsular fraction of the biofilm formed on the mineral surface. Likely, they are involved in the formation of the biofilm matrix mediating the mechanical stability of biofilms and determining its architecture.

EPS produced by *A. hydrophila* contained apolar regions rich in uronic acids. Uronic acids could promote polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (contributing to environmental detoxification). In addition, charged functional groups of uronic acids have the ability to bind metal cations.

FTIR characterization of EPS

FTIR spectra of EPS isolated from *A. hydrophila* are shown in Figure 3. Figure 3A shows the spectrum of EPS for cells grown on ferric citrate as a soluble iron source. Figure 3B displays the spectrum of EPS of cells grown on insoluble gossan mineral. The spectra of colloidal EPS were different in both cases. Colloidal EPS of cells grown with ferric citrate revealed the presence of two very intense bands at 1,621 and 1,380 cm^{-1} . The first band is characteristic of antisymmetric stretching vibrations and the second absorption corresponds to the symmetric stretching vibrations of the COO^- group in carboxylate anions. These bands are associated with the presence of citrate in the medium. *A. hydrophila* uses lactate as an energy source instead of citrate and citrate remains in solution. Moreover, the intense broad absorbance at 3,435 cm^{-1} is attributed to the O–H stretching modes of vibration in the hydroxyl functional group and

N–H stretching vibrations in amides and amines. This broad band at $\sim 3,400 \text{ cm}^{-1}$ appeared also in the colloidal EPS, when cells were grown on gossan mineral. In addition, the absorption peak at 1,631 cm^{-1} corresponds to the amide I band and the band at 1397 cm^{-1} is assigned to the C–N stretching vibration. The two bands at 1,114 and 1,056 cm^{-1} could be assigned to C–O stretching vibration modes of alcohols. The spectrum of colloidal EPS on mineral revealed the presence of various functional groups, typically found in proteins and polysaccharides.

On the other hand, almost negligible differences were noted for the FTIR spectra of the capsular fraction. There, C–O vibrations of oligo- and polysaccharide structures and carboxylate groups were mainly detected. The spectra showed a peak around 3,520 cm^{-1} corresponding to the ‘free’ O–H stretching vibration of the carboxyl group and another at 3,392 cm^{-1} corresponding to the associated O–H vibration. The absorption peak at 2,977 cm^{-1} is assigned to C–H stretching vibration modes in the hydrocarbon chains and at 3,028 cm^{-1} to C–H stretching vibration in alkenes. FTIR analysis of the capsular EPS revealed the presence of three intense bands at 1,630, 1,475 and 1,396 cm^{-1} . The first two are characteristic of amide I and II bands, respectively. The other spectral band at 1,396 cm^{-1} is assigned to the C–N stretching vibration of proteins. In consequence, the most relevant bands in the capsular EPS of *A. hydrophila* are related to the presence of amino acids. Dissimilatory metal-reducing bacteria are an important group of microbes that can directly or indirectly catalyse the reduction of redox-reactive metal ions such as Fe and Mn. In this way, EPS may contain redox active molecules, such as outer membrane cytochromes, capable of facilitating and directing electron transfer (Summers et al. 2010).

The major components in microorganisms have a large number of sharp and characteristic absorption bands in the fundamental infrared region, allowing evaluation of the composition of EPS without separation into individual components. Typical characteristic adsorption bands were not obtained for quantitative analysis because of the overlap of the spectral bands. Nevertheless, quantitative chemical assays of protein and carbohydrate contents were consistent with the different functional groups determined by FTIR measurements (Table 1).

Comparative SEM and CLSM analysis of biofilm structure

Planktonic and sessile cells of *A. hydrophila* were visualized using SEM (Figure 4). The planktonic and the sessile cells showed a marked difference in their general appearance. When *A. hydrophila* cells were grown on a carbon substratum, cells attached to the surface by

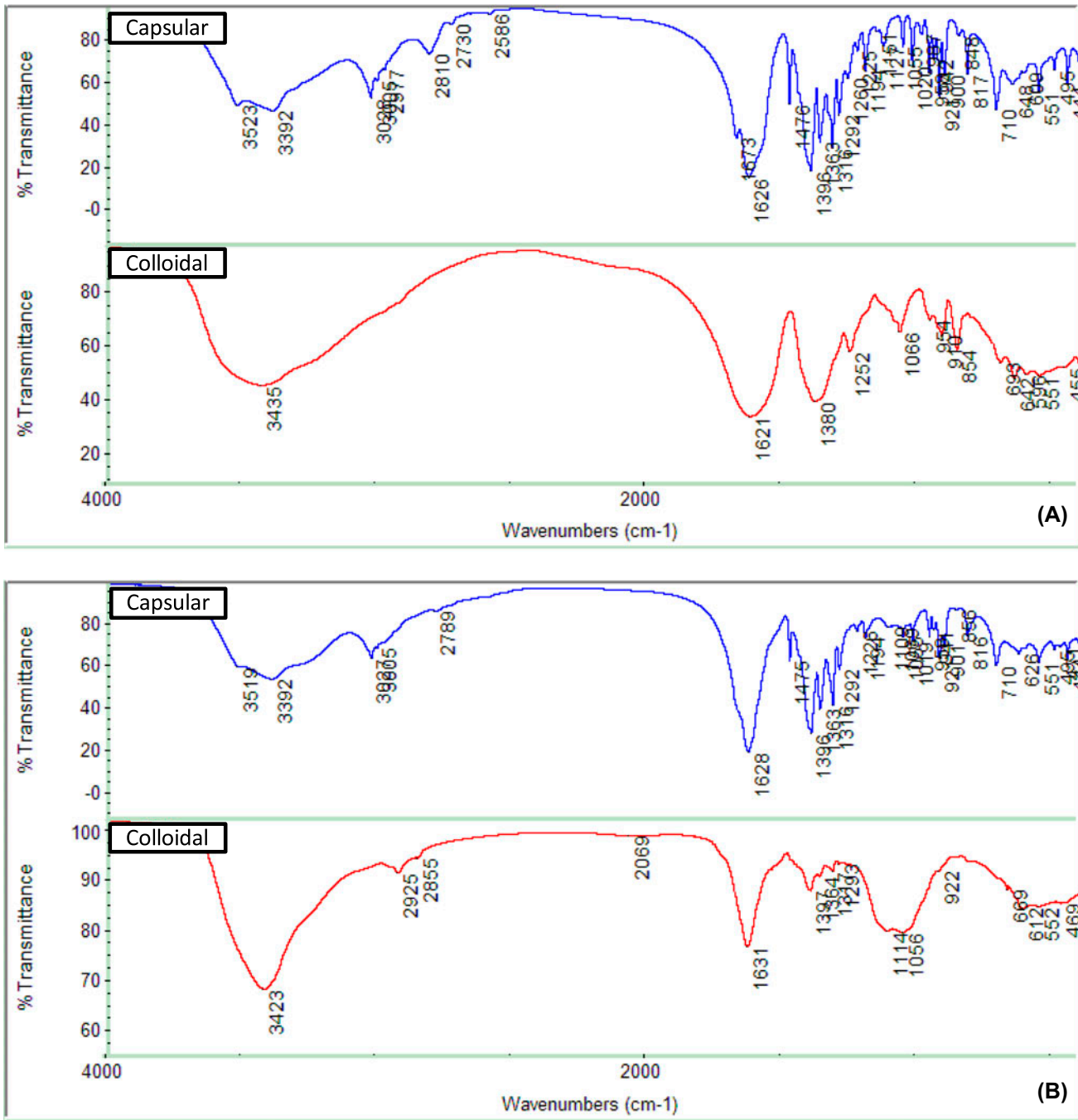


Figure 3. FTIR spectra of EPS (colloidal fraction in red and capsular fraction in blue) of *A. hydrophila* grown: (A) with ferric citrate on carbon fibres; and (B) on gossan mineral as sole electron acceptor.

‘nanowires’ (Figure 4B). These nanowires appeared not only between the cells and the surface but also between (connecting) the cells. Several findings suggest that specialized bacterial pili act as electron conductors to metals as acceptors during respiration (Reguera et al. 2005). Additionally, these nanowires may also play a role in interbacterial signalling. Analyses of bacteria such as

Geobacter sulfurreducens and *Shewanella oneidensis* have revealed this as a possible contact-dependent mechanism of communication (Reguera et al. 2005; Gorby et al. 2006). These strains reduce metals including Fe (III) and Mn(IV) apparently *via* conductance of electrons from the cytoplasmic membrane through nanowire pili. Based on the observation that pili tend to be intertwined

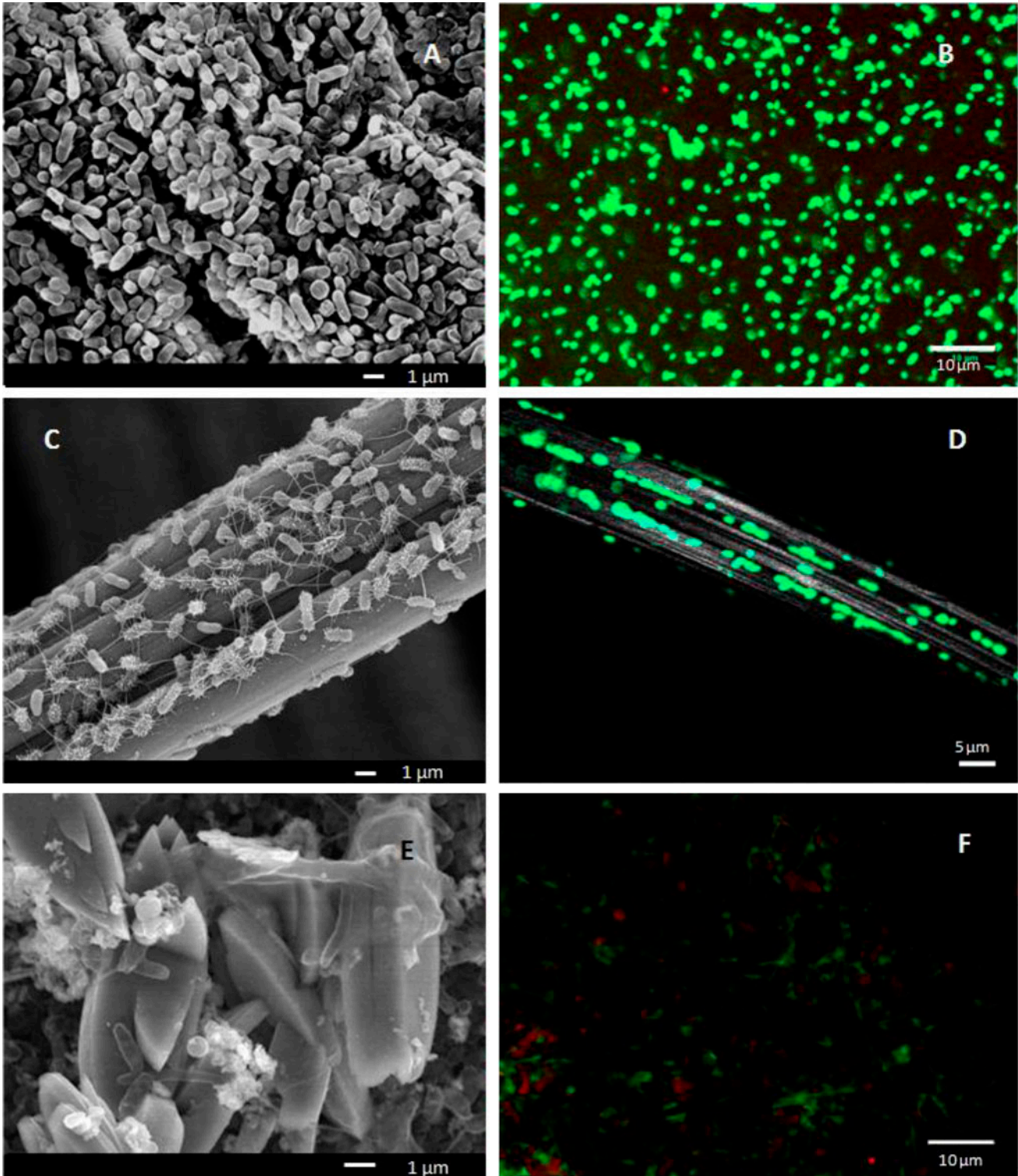


Figure 4. SEM and CLSM images of planktonic cells of *A. hydrophila* (A, D), cells attached to a carbon fibre surface (B, E) and on gossan mineral (C, F). CLSM images of a biofilm region stained with SYTO 9 nucleic acid stain (green) to show the distribution of bacterial cells within the biofilm and with Con A lectin (red) for sugars.

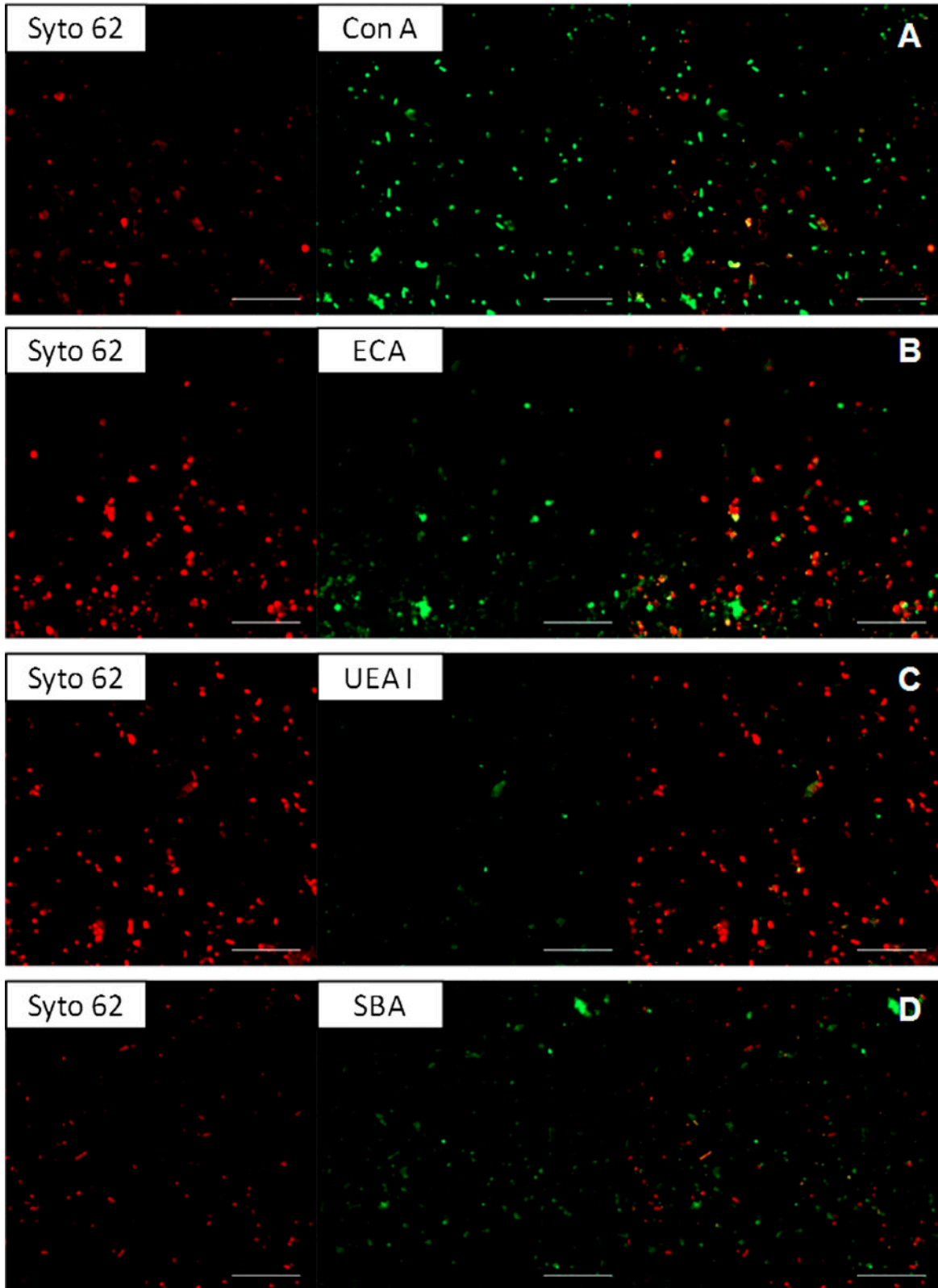


Figure 5. CLSM images of a biofilm region stained with SYTO 62 nucleic acid stain (red) to show the distribution of bacterial cells within the biofilm. The region was also stained with several lectins (green): (A) Con A-FITC, (B) ECA-FITC, (C) UEA I-FITC, and (D) SBA-FITC. The combination of the probes is shown in the images. Scale bars = 10 μ m.

between cells, it is possible that these bacteria are literally plugged into each other *via* nanowire contacts.

SEM observations demonstrated the presence of EPS on solid mineral (Figure 4C). Bacterial biofilm formed a discontinuous coating on the surface of mineral particles and in some places EPS formed organic bridges linking together contiguous particles.

Planktonic cells, cells attached to a carbon surface, cells grown on gossan mineral and biofilms were stained by fluorescently labelled lectins and analysed by CLSM (Figure 4). This technique allows the observation of fully hydrated samples and provides the original shape and structures of the cells and EPS. Nucleic acids were stained with SYTO 9 (green) and fluorescently labelled Concanavalin A lectin (red) was used to visualize exopolysaccharides in a biofilm. Nucleic acid/SYTO 9 staining allowed the observation of the cell distribution in the biofilm. In agreement with the chemical analysis sugars were found only in the EPS developed on the gossan mineral.

Staining of the EPS of a biofilm on a gossan mineral surface using fluorescent lectins

CLSM is the most popular non-destructive technique used to identify visually the different components of the EPS in combination with fluorescent probes (Karunakaran et al. 2011). CSLM coupled with the fluorescently labelled lectins provides a rapid method for easily visualizing and localizing various parts of exopolysaccharides. Previously lectins have been used to obtain useful information on the EPS of pure cultures for analysis of sugars in cell walls (Johnsen et al. 2000; Strathmann et al. 2002; Daubenspeck et al. 2009).

Analysis of biofilm chemistry using CLSM showed the complex chemical composition of the biofilm (Figure 5). Nucleic acid staining showed a uniform distribution of cells on the mineral surface.

Lectins have been used as probes in studies of environmental biofilm systems (Neu et al. 2001; Lawrence et al. 2007). A panel of FITC and TRITC conjugated lectins was tested to analyse the chemical composition of the polysaccharides of *A. hydrophila* in the biofilm formed on the gossan mineral. Only four of the 10 lectins showed a positive response: *Canavalia ensiformis* (Con A), *Erythrina cristagalli* (ECA), *Ulex europaeus* agglutinin I (UEA I), and soybean agglutinin (SBA). Figure 5 shows the combination of two different staining techniques applied sequentially to the same biofilm location: lectins (Con A, ECA, UEA I and SBA) for sugar residues and SYTO 62 for nucleic acids. The results indicated areas that bound none of the probes (black), the presence of cells (red), lectin binding residues (green) or both (yellow).

Con A, ECA and UEA I stained flocs in the culture and did not stain the cells (Figure 5A, B and C). The staining of the living biofilms by Con A could be explained as binding of the lectin to the terminal mannose and terminal glucosyl residues in the EPS secreted by the microorganisms. Mannose and glucose are found in a polysaccharide named Psl, which is involved in the adherence to abiotic and biotic surfaces and in the maintenance of biofilm structure (Ghafoor et al. 2011). During attachment, Psl is anchored to the cell surface in a helical pattern, possibly promoting cell–cell interactions. In addition, other polysaccharides named Pel are rich in glucose and are essential for the formation of biofilms that are attached to a surface (Flemming & Wingender 2010). UEA I stains fucose, while ECA stains galactose and *N*-acetylglucosamine. Extensively studied bacterial fucose-containing EPS include colanic acid, fucogel and clavan (Freitas et al. 2011). Colanic acid is a polysaccharide composed of fucose, glucose, galactose and glucuronic acid. Fucogel is composed of galactose, 4-*O*-acetyl-galacturonic acid and fucose and clavan of glucose, galactose and fucose.

The lectin SBA stained floc structures and also cells (Figure 5D). It gave a positive signal when binding to EPS, indicating the presence of α -linked *N*-acetylglucosamine residues. The identification of SBA as a lectin that binds to EPS likely indicated that some capsular proteins of *A. hydrophila* are glycoproteins containing terminal GalNAc residues as part of their glycan component. Evidence of protein glycosylation in *Pseudomonas aeruginosa* (Brimer & Montie 1998), *Borrelia burgdorferi* (Ge et al. 1998) and *Campylobacter jejuni* (Linton et al. 2002) has been presented.

Conclusions

In this study the composition and distribution of EPS produced by *A. hydrophila* have been investigated. Conventional chemical colorimetric analyses were used to quantify the contents of the EPS, and FTIR spectroscopy allowed a direct analysis of specific components in multi-component mixtures without separation. For the EPS the main components were proteins, indicating possibly their importance for electron transfer reactions. Carbohydrates were found mainly for mineral bound cells likely performing a structural role.

Samples were observed by SEM and CLSM to obtain the shapes and structure of the EPS, as well as its spatial distribution. In addition, CLSM allowed the observation of fully hydrated samples and, combined with a lectin-binding analysis, may be a very useful tool to pre-examine EPS-specific glycoconjugates produced by microorganisms that contribute to biogeochemical processes of iron reduction.

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