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Characterization of extracellular polymers synthesized by tropical intertidal biofilm bacteria

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Abstract:

This study was performed to determine the potential of tropical intertidal biofilm bacteria as a source of novel exopolymers (EPS).

Methods and Results: A screening procedure was implemented to detect EPS-producing biofilm bacteria. Isolates MC3B-10 and MC6B-22, identified respectively as a *Microbacterium* species and *Bacillus* species by 16S rDNA and cellular fatty acids analyses, produced different EPS, as evidenced by colorimetric and gas chromatographic analyses. The polymer produced by isolate MC3B-10 displays significant surfactant activity, and may chelate calcium as evidenced by spectroscopic analysis.

Conclusions: Polymer MC3B-10 appears to be a glycoprotein, while EPS MC6B-22 seems to be a true polysaccharide dominated by neutral sugars but with significant concentrations of uronic acids and hexosamines. EPS MC3B-10 possesses a higher surfactant activity than that of commercial surfactants, and given its anionic nature, may chelate cations thus proving useful in bioremediation. The chemical composition of polymer MC6B-22 suggests its potential biomedical application in tissue regeneration.

Significance and Impact of the Study: This is the first report of a *Microbacterium* species producing EPS with surfactant properties, which expands our knowledge of the micro-organisms capable of producing these biomolecules. Furthermore, this work shows that tropical intertidal environments are a nonpreviously recognized habitat for bioprospecting EPS-producing bacteria, and that these molecules might be involved in ecological roles protecting the cells against dessication.

INTRODUCTION

Bacteria live predominantly associated with surfaces as biofilm communities in natural and man-made environments in both terrestrial and aquatic settings (Costerton *et al.* 1981; Guezennec *et al.* 1998; Stoodley *et al*. 2002). This life style has often been interpreted as an ecological strategy to cope with physical and chemical stress. Marine biofilms have been shown to play key ecological roles sustaining populations of sessile invertebrate grazers (Thompson *et al.* 2000) and cycling carbon and nitrogen (Magalhães *et al.* 2003).

Recently, marine biofilms have also attracted attention due to their biotechnological potential. Vincent *et al.* (1994) and Raguenes *et al.* (1996) assessed the potential of biofilm-derived bacteria from deep-sea hydrothermal vents as producers of new exopolysaccharides, some with potential application in the food sector, while other exhibit a bioactive behaviour; bioactive polysaccharides are capable of eliciting physiological activities from the molecular to the organismal level ( ). More recent studies showed that a novel bacterium, *Paracoccus zeaxanthinifaciens* subsp. *payriae* isolated from a microbial mat (biofilm-like communities) produced bioactive exopolysaccharides with high sulfate content (Raguenes *et al.* 2004), while a psychrophilic *Pseudoalteromonas* species isolated from sea-ice microbial communities in the Antarctic, produced a highly anionic extracellular polymers (EPS) (Mancuso *et al.* 2004). A common feature of these studies is that they have been carried out using bacteria isolated from biofilm communities exposed to extreme conditions. These studies have hypothesized that environmental stress such as high barostatic pressure, large temperature gradients and high salinity, among other factors, select for novel microorganisms capable of producing unusual exopolysaccharides.

On the other hand, limited bioprospecting efforts have been undertaken to isolate novel exopolysaccharide-producing microorganisms from other non-previously recognized extreme marine habitats. Intertidal rocky shores are exposed to a combination of harsh factors such as wave action, thermal and desiccation stress, UV exposure and nutrient depletion (Menge and Branch 2001). Desiccation appears to induce the production of copious amounts of exopolymers, presumably of a highly hygroscopic nature, by biofilms as an ecological strategy to cope with this type of stress (De Winder *et al*. 1990; Potts 1994; Ortega-Morales *et al*. 2001). Since atmospheric exposure of intertidal habitats after emersion also produces desiccation stress on extant microbial communities, it is reasonable to hypothesize that these habitats are likely to harbour diverse bacteria capable of producing polysaccharides. Despite their importance, however, to our knowledge only one report has been published to date describing the potential of intertidal bacteria as exopolymer-producers (Boyle and Reade 1983). Here we report the results of a partial chemical characterisation of EPS produced by intertidal biofilm bacteria isolated from a range of natural and artificial surfaces exposed to a subtropical intertidal rocky shore in southern Gulf of Mexico (Campeche, Mexico), a more detailed chemical study of selected polymers, along with the molecular identification of the producing isolates.

MATERIALS AND METHODS

Isolation of bacteria

Leaves of the seagrass *Thalassia testudinum*, copper coupons and rock samples were collected from a pristine rocky intertidal shore in the State of Campeche, Southern Gulf of Mexico. This type of shore is common in the Yucatan Peninsula. Samples of leaves and rock chips showing heavy biofilm growth were aseptically recovered in sterile plastic bags, chilled on ice and

transported to the laboratory. Copper coupons (2 cm^2) were treated following the same procedure, except that these coupons were placed 15 d before the sampling period to allow for biofilm development. Copper is a bioactive substratum shown to select for exopolymer-producing microorganisms (Marszalek *et al.* 1979; Guezennec 2002). Once in the laboratory, the samples were gently washed with sterile seawater to remove loosely attached bacteria and placed in 10 ml of sterile seawater and vortexed for one minute to obtain cell suspensions. These suspensions were serially diluted and plated on Marine Agar (MA, Difco Laboratories, Detroit, MI). Plates were then incubated at 30°C for 5 d and checked daily to pick by a random procedure representative colonies which were then successively restreaked for purification. Isolates were checked by microscopy and although this procedure does not ensure pure cultures nor allows dereplication of isolates, it has been used successfully in the past to obtain pure exopolysaccharide-producing bacterial isolates. Strains were preserved as glycerol suspensions (20%, w/v) at -80°C. Working cultures were maintained on Marine Agar 2216 slants at 4°C.

Screening for EPS synthesis

In a first screening, thirty-four strains were screened for their ability to synthesize EPS in Marine agar 2216 (MA) plates amended with 3% (w/v) glucose (MA + 3 % glucose). Calibrated loops were used to streak ~30 µl of overnight cultures (grown on MB) on MA + 3% glucose plates. The plates were incubated at 30°C for 48 h. Mucoid (slimy) colonies, indicative of their ability to produce exopolymeric substances (De Vuyst and Degeest 1999; Fusconi and Godinho 2002; Maugeri *et al*. 2002), were selected for further screening. Control cultures were performed with MA plates without the glucose amendment (Raguenes *et al*. 1997).

In a second step, the presumably EPS-producing strains (11 isolates) were reinoculated (~30 µl) on MA + 3% glucose plates and incubated as described above. A marine biofilm forming bacterium *Halomonas marina* CCUG16095 obtained from the Culture Collection, University of Göteborg, Sweden, was used a positive control organism. This bacterium has been shown to produce significant concentrations of EPS in agar and liquid cultures (Ford and Mitchell 1992; Chan-Bacab and Ortega-Morales 2005). The bacterial biomass was then carefully scraped from the agar surface by means of a spatula and split in two aliquots. One aliquot was used to determine wet weight, while the remaining fraction was transferred to a tube containing 5 ml of 3.5% NaCl solution (w/v). This latter fraction containing the bacterial suspension was then vortexed for 5 min and centrifuged at 10,000 x *g* for 5 min. The cell pellet was discarded and the supernatant was then mixed with two volumes of cold absolute ethanol. The precipitated EPS fraction was assayed using the Dubois method with glucose as standard. The content of polysaccharides per unit dry weight of bacterial biomass was then determined, assuming a 95% water content in biofilms (Zhang *et al.* 1998).

Production and preliminary chemical characterisation of exopolymers.

After the screening procedure, 7 strains were retained for exopolymer (EPS) production, from which four isolates were selected on the basis of the highest amount of polysaccharide produced per unit colonial biomass on marine agar plates (MC1B-03, MC3B-10, MC6B-28 and MC1B-32), while three additional bacterial isolates (MC6B-02, MC3B-13 and MC6B-22) were also studied since they showed good growth in MB (Table 1). EPS production was performed at 30°C in 1-l Erlenmeyer flasks containing 500 ml of marine broth amended with glucose (30 g l^{-1}). Batch fermentation was started by inoculating 50 ml of a suspension of cells grown overnight in the same culture medium. The flasks were then shaken at 200 rpm (LAB-LINE Instruments, INC,

IL, USA) at 30°C for 48 h. Bacterial growth was determined spectrophotometrically at 520 nm from aliquots that were removed at regular intervals.

After the cultures reached the exponential phase of growth (24-36 h), culture broths were heated at 100°C for 15 min to inactivate the enzymes capable of degrading the polymer (Cerning *et al*. 1994). Cells were then removed by centrifugation at 4,000 x *g* for 30 min at 4°C and filtered through 0.47 µm pore diameter HVLP filters. The supernatant was allowed to stand overnight at 4°C; the EPS were then precipitated by adding 2 volumes of cold absolute ethanol. The EPS were collected by centrifugation 4,000 x *g* for 30 min at 4°C and redissolved in a small volume of distilled water, to repeat the precipitation procedure as described above. It was subsequently dialyzed (molecular weight cut-off 6,000 to 8,000 Da) against deionized water for 48 h, reprecipitated and dried at 40°C and estimated gravimetrically. Protein content was determined following the method proposed by Lowry *et al.* (1951) using albumin as the standard protein. Hexuronic acid concentrations were determined by the *m*-phenylphenol method (Blumenkrantz and Asboe-Hansen 1973), with glucuronic acid as the standard, while total neutral carbohydrate content was determined by the orcinol-sulfuric method (Tillmans and Philippi 1929; Rimington 1931) using a standard of a 1:1 molar ratio mixture of mannose-galactose.

Given the anionic composition of polymers produced by strains MC3B-10 and MC6B-22, as revealed by colorimetric analyses, their monosaccharide composition was determined as described below. The producing bacterial isolates were identified by sequencing of 16S rDNA and whole cellular fatty acid analyses. Since only the isolate MC3B-10 yielded relatively high concentration of polymer in our laboratory conditions (~2 g l^{-1}), the surfactant activity and additional spectroscopic analyses were performed on this single polymer.

Characterization of EPS MC3B-10 and MC6B-22

Monosaccharide composition. The monosaccharides were analysed after hydrolysis by acid methanolysis of the polymers with MeOH/HCl (2 N) during 4 h at 100°C and subsequent GC analyses of peracetylated derivatives of trimethyl-sylil derivatives. Erythritol was used as internal standard. The analytical procedure was performed using a Fisons instrument (GC 8000 series) fitted with a WCOT fused silica CP-SIL 5CB (60 m x 0.25 mm) with a temperature gradient from 50°C to 120°C (at 20°C min^{-1}) and from 120°C to 250°C (at 2°C min^{-1}) and hydrogen as carrier gas. The molar ratios of monosaccharides were determined according to Kamerling *et al.* (1975) and Montreuil *et al.* (1986).

Assessment of surfactant activity. The surfactant activity of EPS MC3B-10 was determined as previously reported (Bouchotroch *et al*. 2000). Briefly, equal volumes of EPS solutions [1% (w/v) in deionized water] and aliphatic hydrocarbons of increasing carbon chain length were added to 12-mm-diameter glass tubes and vigorously mixed using a vortex. The tubes were allowed to stand for 24 h. Surfactant activity was expressed as the percentage of the total height occupied by the emulsion. The hydrocarbons (*n*-hexane, *n*-octane and *n*-hexadecane) and control surfactants (Tween 80 and Triton X-100) were purchased from Sigma (St. Louis, MO).

X-ray Photoelectron (XPS) and Fourier Transform Infrared Spectroscopy analyses. Fourier transform infrared spectroscopic analysis (FT-IR) was performed according to Suci *et al.* (1997). Spectra were acquired with a 4 cm^{-1} resolution in the 4000-400 cm^{-1} region, using a Nicolet Magna 460 FTIR spectrometer with a deuterade triglycine sulfate (DTGS) detector in the

transmission mode. Compressed tablets were prepared by mixing 2 mg of EPS with 100 mg of KBr. Spectra were corrected for KBr background using the OMNIC software.

XPS analyses were performed in a Perkin-Elmer PHI 560/ESCA-SAM system, equipped with a double-pass cylindrical mirror analyzer, with a base pressure of 1×10^{-9} Torr. Argon ion sputtering was performed with 4 keV energy ions and 0.36 μA/cm^2 current beam, yielding to about 3 nm/min sputtering rate. All XPS spectra were obtained after 5 min of Ar^+ sputtering. The utilized low current density in the ion beam and short cleaning time reduce possible drastic modifications in the stoichiometry of the surface. For the XPS analyses, sample was excited with 1486.6 eV energy AlKα X-ray. XPS spectra were obtained under two different conditions: (i) a survey spectrum mode of 0-600 eV, and (ii) a multiplex repetitive scan mode. No signal smoothing was attempted and a scanning step of 1 eV/step and 0.2 eV/step with an interval of 50 ms was utilized for survey and multiplex modes, respectively. The spectrometer was calibrated using the Cu 2p3/2 (932.4 eV) and Cu 3p3/2 (74.9 eV) lines. Binding energy calibration was based on C 1s at 284.8 eV.

Bacterial identification

16S rDNA sequence analysis. The 16S rDNA genes of isolates MC3B-10 and MC6B-22 were analysed. DNA was extracted, using the Wizard Genomic DNA Purification kit, according to the manufacturer's protocol (Promega). The 16S rDNA gene was amplified by PCR using universal primers Fd1 (5'-CAGAGTTTGATCCTGGCTCAG-3') and R6 (5'-TACGGTTACCTTGTTACGAC-3') for strain MC3B-10 and the primers Fd1 and Rd1 (5'-AAGGAGGTGATCCAGCC-3') (Winker and Woese 1991) for strain MC6B-22. The PCR

mixture contained 50 µl final volumen: 5.0 µl of 10X buffer, 5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mmol l^{-1} dNTP mixture, 0.5 µl of a 50 pmol $µl^{-1}$ solution of each primer, 0.5 µl of Taq polymerase (5 U $µl^{-1}$), qsp. water. The DNA amplification was performed using a Perkin Elmer Gene Amp PCR, System 2400. The PCR program was as follows: 95°C for 1 min, 30 cycles of 20 s at 95°C, 30 s at 55°C, 1.30 min at 72°C, and final extension of 5 min at 72°C. PCR products were visualized under UV light after electrophoresis on a 0.8% (w/v) agarose gel containing ethidium bromide. PCR products were cloned using the pGEM-T-easy cloning kit and chemically competent *Escherichia coli* JM109 cells, according to the manufacturer's protocol (Promega). The clone library was screened by direct PCR amplification from a colony using the vector specific primers SP6 (5'-ATTTAGGTGACACTATAGAA-3') and T7 (5'-TAATACGACTCACTATAGGG-3') and the same reaction conditions as described above, was used. A plasmid containing the right length insert was isolated using the kit Wizard Plus SV Minipreps DNA Purification System (Promega) as described in the protocol. Purified plasmids were sent for sequencing to Genome Express (Grenoble, France).

Sequence analysis. The nucleotide sequences of the 16S rDNA genes (about 1400 nucleotides each) were aligned using Clustal W program, version 1.5 (Thompson *et al*. 1994) and then manually adjusted. Reference sequences were obtained from the Ribosomal Database Project (Maidak *et al*. 2001) and GenBank database (Benson *et al*. 1999). Positions of sequence and alignment uncertainty were omitted from the analysis. Phylogenetic inference was obtained by using a Bayesian approach, a model-based phylogenetic method, as implemented in MRBAYES 3.01. This program uses the Bayes theorem and the models of Monte Carlo Markov Chains (MCMC) and the General Time Reversible (GTR) nucleotide substitution model (Huelsenbeck and Ronquist 2001). The analysis was run for 1 x 10^6 generations and the trees sampled after

reaching chain stationary (the “burn-in”) were used in a majority-rule consensus tree. Full 16S rDNA sequences of 2 isolates were deposited into the GenBank database under the accession numbers AY833570 (MC3B-10) and AY833573 (MC6B-22).

Cellular fatty acid methyl ester (FAME) analysis. These strains were grown and the phospholipids were extracted and derivatized for FAME analysis using gas-liquid chromatography following the manufacturers’ instructions (Sherlock Microbial Identification System; MIDI, Inc., Newark, Del.).

RESULTS

Screening of EPS-producing bacterial isolates

A total of 34 bacterial isolates, randomly taken from MA plates inoculated with different types of biofilms from an intertidal tropical rocky shore, were screened for EPS production on both Marine Agar (MA) and MA amended with glucose (MA + glucose). Most of the mucoid isolates were obtained from epilithic (rock surface) biofilms. Out of these 34 isolates, 11 strains developed as mucoid colonies; this was particularly the case of colonies grown on MA + glucose. These 11 isolates were obtained from copper surfaces (41%), epilithic biofilms (27%) and epiphytic biofilms (27%), respectively.

Seven isolates (MC6B-02, MC1B-03, MC3B-10, MC3B-13, MC6B-22, MC6B-28 and MC1B-32) showed a more abundant growth on agar plates than the rest of the isolates and/or grew well

in liquid medium. No correlation was seen between isolates exhibiting good growth in MA plates and marine broth (Table 1).

These isolates synthesized varying concentrations of EPS, whose chemical composition also varied (Table 2). Protein levels were in general high, except for strain MC6B-22 (8.9%). Neutral sugars also tended to dominate in the sugar profiles of EPS from most isolates. Significant concentrations of acid sugars were seen in EPS from isolate MC3B-10 (9.1%) and MC6B-22 (14.7 %). Hexosamine content was highly variable among the isolates, but strain MC6B-22 produced an exopolysaccharide with unusually high concentrations of this monosaccharide (21.1%).

Bacterial identification

The identification of isolates MC6B-22 and MC3B-10 was carried out using both 16S rDNA sequencing and whole-cell fatty acid analyses. Both isolates were Gram positive organisms that displayed mucoid colonial phenotypes. Strain MC3B-10 displayed a strong orange pigmentation when grown on MA plates. However, an enhanced phenotype of the colonies exhibiting an even stronger pigmentation, were seen when they were grown on plates of MA amended with glucose. In turn, isolate MC6B-22 produced opaque, irregular colonies.

Phylogenetic analysis based on the 16S rDNA sequences placed these strains as members of the genus *Microbacterium* (MC3B-10) and *Bacillus* (MC6B-22). Strain MC3B-10 was closely related to *Microbacterium trichotecenolyticum, M. flavescens* and *M. kitamense* (Fig. 1), with similarity values ranging from 97 to 99%. On the other hand, isolate MC6B-22 was identified as

303 a close relative of *Bacillus mojavensis* and *B. subtilis* (Fig. 2), with similarity values up to 99%.
304 Definitive identification of these isolates will require quantitative DNA-DNA hybridization
305 analysis (Raguenes *et al*. 2004).
306
307 **Characterization of exopolymers**
308 The screening of isolates implemented in this study showed that strains MC3B-10 and MC6B-22
309 presented the most interesting properties. Therefore, they were retained for further
310 characterization studies. GC analysis of monosaccharides as per-*O*-trimethylsilyl
311 methylglycosides of these polymers is given in Table 3. Glucose predominated in the neutral
312 sugar fraction of both polymers, although this sugar represented a higher proportion (25%) of the
313 total monosaccharides in the EPS MC3B-10. Lower levels of galactose and mannose were
314 detected in both EPS. Rhamnose was found only in EPS MC3B-10 in small amounts. Both
315 polymers possessed uronic acids (galacturonic acid and glucuronic acid) and hexosamines (N-
316 acetyled glucosamine), but the concentrations of the latter compound were significantly higher in
317 the EPS synthesized by isolate MC6B-22.
318
319 The FTIR spectrum of the polymer produced by isolate *Microbacterium* sp. MC3B-10 displayed
320 a broad O-H stretching band at 3421 cm^{-1} and at an intense band at 1070 cm^{-1}, typical of
321 carbohydrates. In addition, bands at 1650 and 1550 cm^{-1} were also detected. No doublet at 1250
322 and 1230 cm^{-1}, indicative of the presence of ester sulfate groups, was seen (Fig. 3). On the other
323 hand, the XPS spectrum (Fig. 4) of this polymer shows the O 1s (533 eV), N 1s (401 eV), Ca
324 2p3/2 (349 eV) and C 1s (287 eV) as core level principal peaks. Also, Ca 2s (441 eV), Ca 3s (46
325 eV) and Ca 3p (27 eV) secondary peaks were detected.
326

EPS MC3B-10 was shown to exhibit surfactant activity against aliphatic hydrocarbons. This polymer exhibited a higher activity than commercial surfactants such as Triton X-100 and Tween 80. This trend was more evident with hydrocarbons of long chain length (*n*-hexadecane) than with short-chained hydrocarbons (*n*-hexane; Fig. 5).

DISCUSSION

This study expands our knowledge on the ability of intertidal bacteria to synthesize EPS, since a previous study reported only EPS-producing bacterial from cold coasts (Boyle and Read 1983). Most of the isolates obtained in this study exhibited mucoid colonial phenotypes, when plated on Marine agar (MA), and presented an enhanced (more) viscous morphology, when grown on MA supplemented with glucose. Two bacterial isolates, identified by 16S rDNA sequencing and cellular fatty acid analysis as a *Microbacterium* species and a *Bacillus* species, were further selected on the basis of their ability to grow well both in solid (agar) and liquid marine media and because they produced polymers with significant uronic acid and hexosamine levels as indicated by colorimetric analysis.

These EPS differed in their chemical composition, as shown by colorimetric and gas chromatographic (GC) analyses. Polymer MC3B-10 had high levels of protein (36%), a finding confirmed by FTIR data which showed intense bands at 1640 and 1550 cm^{-1}, typical of C=O stretching in secondary amides (amide I) and N–H deformation and C-N stretching in –CO–NH– (amide II) in proteins (Suci *et al.* 1997; Omoike and Chorover 2004). In contrast, polymer synthesized by isolate *Bacillus* sp. MC6B-22 had lower amounts of proteins. Another important differing feature between both EPS was the higher concentration of neutral sugars in polymer MC3B-10 and hexosamines (~10 fold) in polymer MC6B-22. These findings suggest that the

exopolymer produced by the strain MC3B-10 is not a polysaccharide, but a glycoprotein. This is further supported by the detection of significant surfactant activity and because this polymer was not completely soluble in distilled water. Amphipathic molecules including glycoproteins possess surfactant activity that may render them potential candidates for environmental and medical applications (Rosenberg and Ron 1999; Cameotra and Markkar 2004). Reinforcing this finding, XPS analyses showed nitrogen and carbon as dominant elements in the elemental chemical profile. In addition, the N/C ratio was 0.14, which is close to the reported ratio of 0.16 for a mixture of protein and polysaccharide (Rubio 2002). Interestingly, this analysis also showed that calcium was associated with this polymer. This signal was detected in freeze-dried amorphous polymer samples (Fig. 4) that were exhaustively washed with deionized water. This suggests that this element was sequestered from the bulk culture medium and points toward a likely chelating property, which is congruent with the anionic composition (presence of hexuronic acids) of this EPS, although electron donating moieties such as carbonyls and hydroxyls in polysaccharides may also chelate cations such as Ca^{2+} (Perry *et al.* 2004). Although more work is needed to define the chelating properties of this polymer, our finding suggests its application as a new biosorbant (Guezennec *et al.* 2002).

On the other hand, the low levels of protein in polymer MC6B-22 and the considerable amounts of hexuronic acids, especially glucuronic acid and hexosamines (glucosamine-*N*-acetylated and galactosamine-*N*-acetylated) suggest that it is a polysaccharide with promising biomedical activities. Hyaluronic acid (HA) is a polymer naturally found in connective tissue and is composed of a repeating disaccharide unit of *N*-acetylglucosamine and glucuronic acid, that has potential applications in wound healing and angiogenesis (Cen *et al.*2004). In addition, tissue

regeneration (bone healing) properties have been found in a new hyaluronic acid-like bacterial exopolysaccharide (Zanchetta *et al*. 2003a; Zanchetta *et al*. 2003b).

It is interesting to note that the chemical composition of EPS from both isolates is different from other species from the same genera. Matsuyama *et al.* (1999) showed that a new *Microbacterium* species, *M. kitamiense* produced both soluble and insoluble EPS. Analysis of these EPS showed that they contained neither protein nor uronic acids, significantly differing from the EPS produced by *Microbacterium* MC3B-10. Similarly, polymer produced by our *Bacillus* sp. MC6B-22 contained aminosugars and uronic acids. In contrast, Mança *et al.* (1996) reported the synthesis of a sulfated heteropolysaccharide composed exclusively of mannose and glucose in a *Bacillus thermoantarcticus* strain. This suggests that exopolymer production and composition in biofilms is a species-specific process, although the influence of culture medium cannot be ruled out (Sutherland 2001).

Microbacterium and *Bacillus* species have been shown to form biofilms and inhabit other marine environments, including sediments, invertebrate tissues (surfaces) and artificial aquacultural ponds (Wicke *et al.* 2000; Lang *et al.* 2004). However, the *Bacillus* isolate MC6B-22 obtained from a biofilm developed on a copper coupon, to our knowledge is the first report of a tropical intertidal bacterium closely related to *Bacillus mojavensis* and *B. subtilis*, organisms considered of terrestrial origin. We cannot establish at this point, however, if this bacterium is a true marine bacterial isolate or if it is a transient intertidal colonizer that originated from nearby terrestrial environments. It is also interesting to note that isolate *Microbacterium* MC3B-10 produced an EPS with significant surfactant activity, which was in fact higher than that exhibited by commercial compounds. These EPS may participate in structuring epilithic biofilms conferring

them mechanical stability (Mayer *et al*. 1999) and protect bacterial populations from desiccation (De Winder *et al.* 1990; Potts 1994; Ortega-Morales *et al.* 2001), ecological roles that are congruent with the stress imposed by intertidal areas (Menge and Branch 2001). In addition, surfactant EPS may also enable the component cells embedded in epilithic biofilms to solubilize and utilize substrates which would otherwise be inaccessible (Sutherland 2001; Ortega-Morales *et al.* 2001). In a previous study, we showed that significant levels of EPS were found associated with epilithic biofilms in the Gulf of Mexico (Narváez-Zapata *et al.* 2005).

Further genetic studies (DNA-DNA hybridization) and chemical (NMR), as well as biological analyses (bioassays) are needed to fully assess the biotechnological potential of polymers MC3B-10 and MC6B-22 in particular in biomedical and environmental fields. Also more detailed studies are required to determine the ecological role that these compounds may play in the survival and persistence of these bacteria in naturally occurring intertidal biofilms.

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631

631 **Table 1** Growth and production of EPS in liquid marine broth (MB) of selected intertidal biofilm bacteria

Strain	Growth on solid medium (µg polysaccharide per mg dry weight of colony biomass)	Growth in liquid medium (O.D.)	EPS yield ($mg\ l^{-1}$)
MC6B-02	348 ± 12*	5.4	97.2 ± 20.9
MC1B-03	768 ± 12	6.6	228.0 ± 15.8
MC3B-10	672 ± 48	8.9	2229.0 ± 174.1
MC3B-13	468 ± 72	13.4	190.4 ± 52.6
MC6B-22	360 ± 12	7.0	344.8 ± 11.3
MC6B-28	648 ± 24	2.5	195.8 ± 86.3
MC1B-32	684 ± 6	1.8	152.6 ± 57.4
H. marina	751 ± 32	7.1	1804.0 ± 58.1

632 * Means of three determinations ± S.D.

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635

636 **Table 2** Chemical composition of EPS synthesized by intertidal biofilm bacterial isolates.

Isolate	Composition (%)			
	Proteins	Neutral sugars	Hexuronic acids	Hexosamines
MC6B-02	26.41 ± 4.2*	32.81 ± 2.9	2.59 ± 0.5	8.54 ± 3.1
MC1B-03	43.50 ± 2.9	40.44 ± 4.9	2.53 ± 0.2	7.58 ± 0.0
MC3B-10	36.17 ± 2.8	45.02 ± 3.9	9.10 ± 1.6	2.65 ± 1.0
MC3B-13	46.98 ± 3.5	22.01 ± 3.3	2.44 ± 0.0	9.97 ± 2.1
MC6B-22	8.90 ± 3.2	5.56 ± 2.2	14.67 ± 2.0	21.15 ± 7.5
MC6B-28	23.62 ± 5.7	32.74 ± 3.9	1.23 ± 0.1	15.15 ± 5.5
MC1B-32	25.64 ± 2.4	31.80 ± 17.6	1.75 ± 1.2	1.50 ± 1.4

637 * Means of three determinations ± S.D.

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639

640 **Table 3** Monosaccharide composition (molar ratios) of the polymers produced by isolates *Microbacterium* sp.
641 MC3B-10 and *Bacillus* sp. MC6B-22.

Isolate	Man	GlcUA	GalUA	Gal-N-Ac	Glc-N-Ac	Glc	Rha	Gal
MC3B-10	6.6	3.7	3.6	-	3.7	25.8	1.7	9.6
MC6B-22	3.1	6.8	1.1	7.8	4.0	4.7	-	-

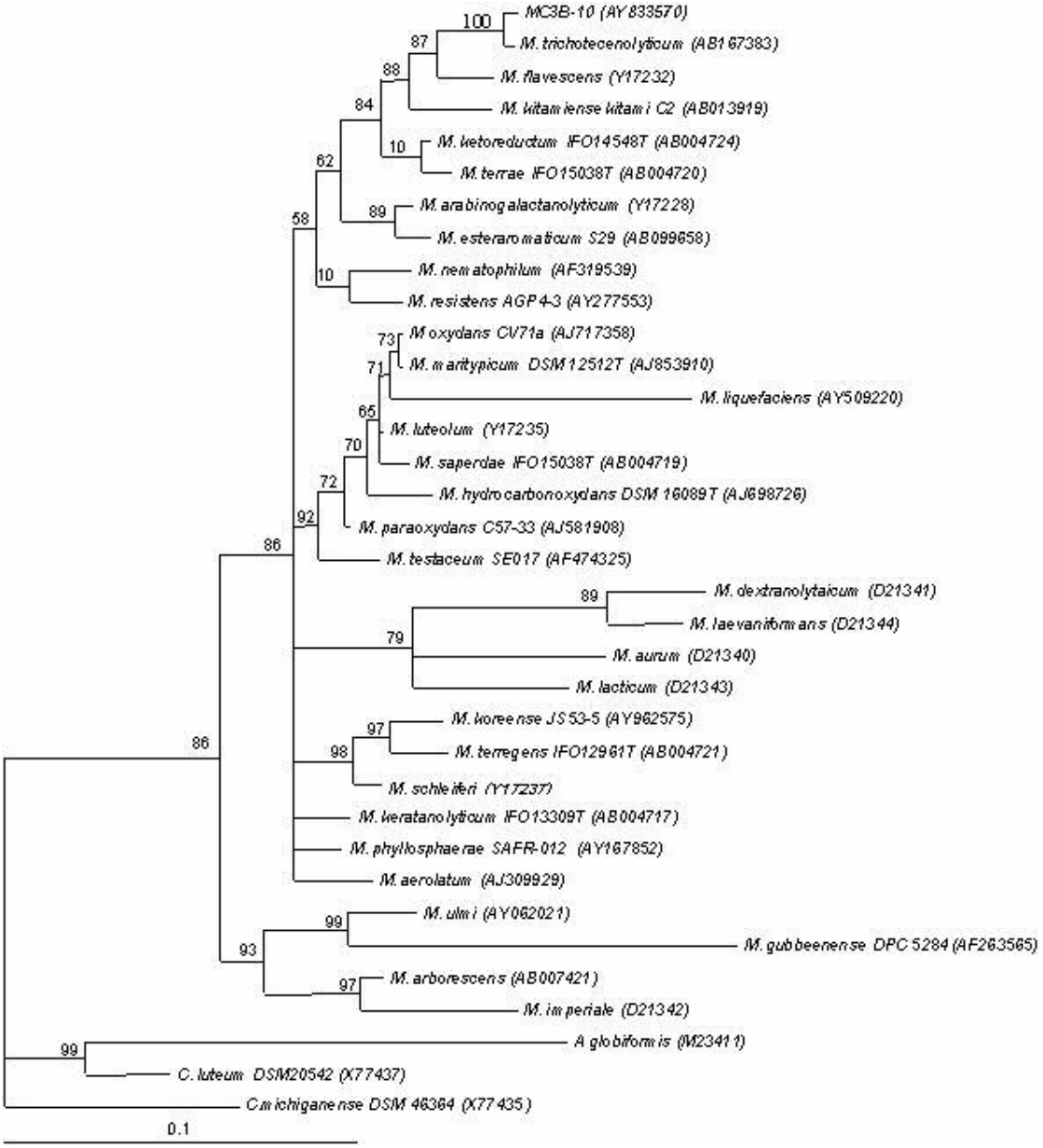

Fig. 1 Majority rule consensus tree showing the results from Bayesian analysis based on 16S rDNA sequence data, indicating the position of isolate MC3B-10 (AY833570) among members of the genus *Microbacterium*. Accession numbers of 16S rDNA gene sequences of reference organisms are shown in parenthesis. Numbers above the branches denote posterior probabilities to percentage converted. Branches with a posterior probability of < 0.5 have been collapsed. *Curtobacterium luteum* and *C. michiganense* were used as outgroups.

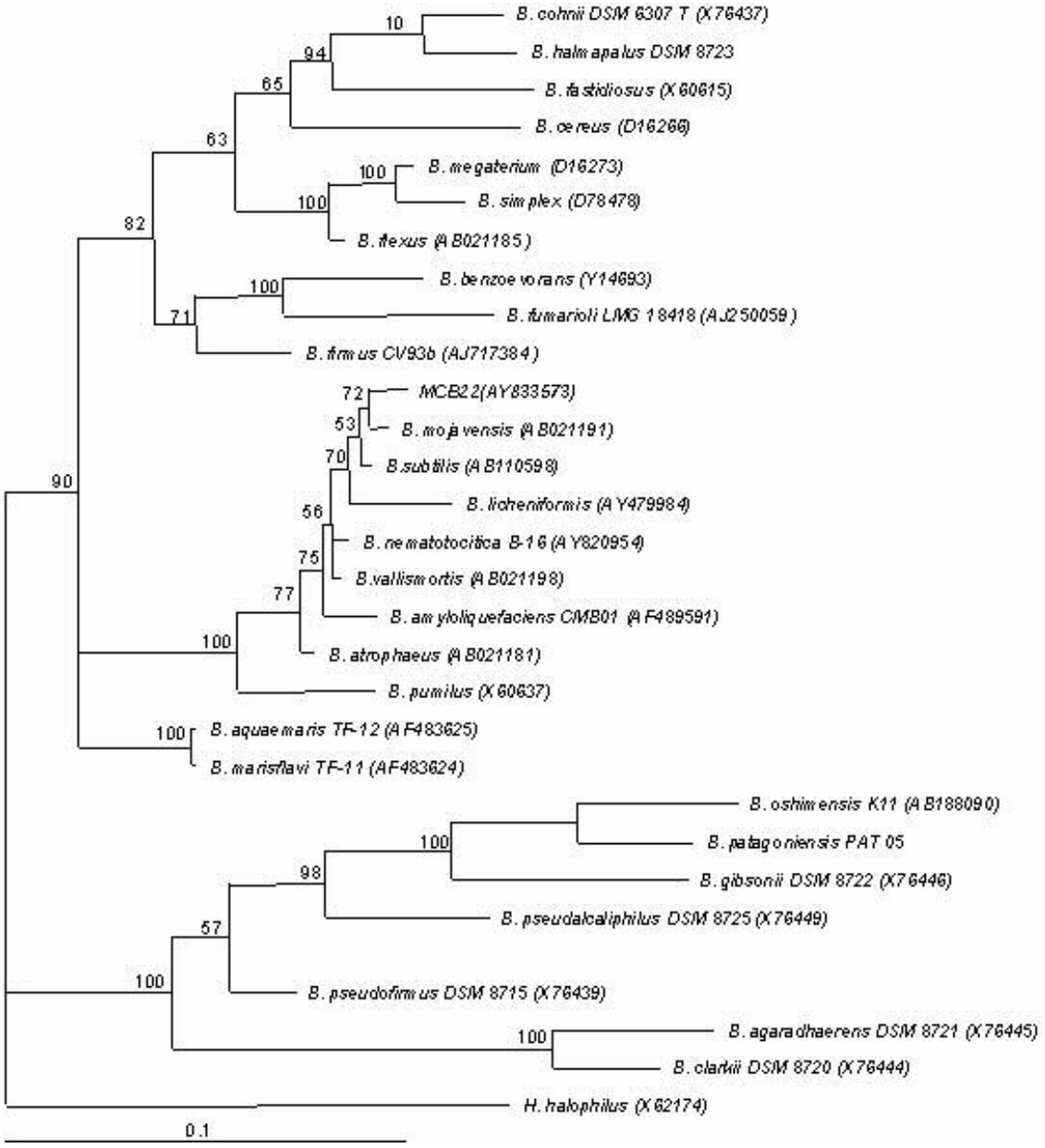

Fig. 2 Majority rule consensus tree showing the results from Bayesian analysis based on 16S rDNA sequence data, indicating the position of isolate MCB22 (AY833573) among members of the genus *Bacillus.* Accession numbers of 16S rDNA gene sequences of reference organisms are shown in parenthesis. Numbers above the branches denote posterior probabilities to percentage converted. Branches with a posterior probability of < 0.5 have been collapsed. *Halobacillus halophilus* was used as outgroup.

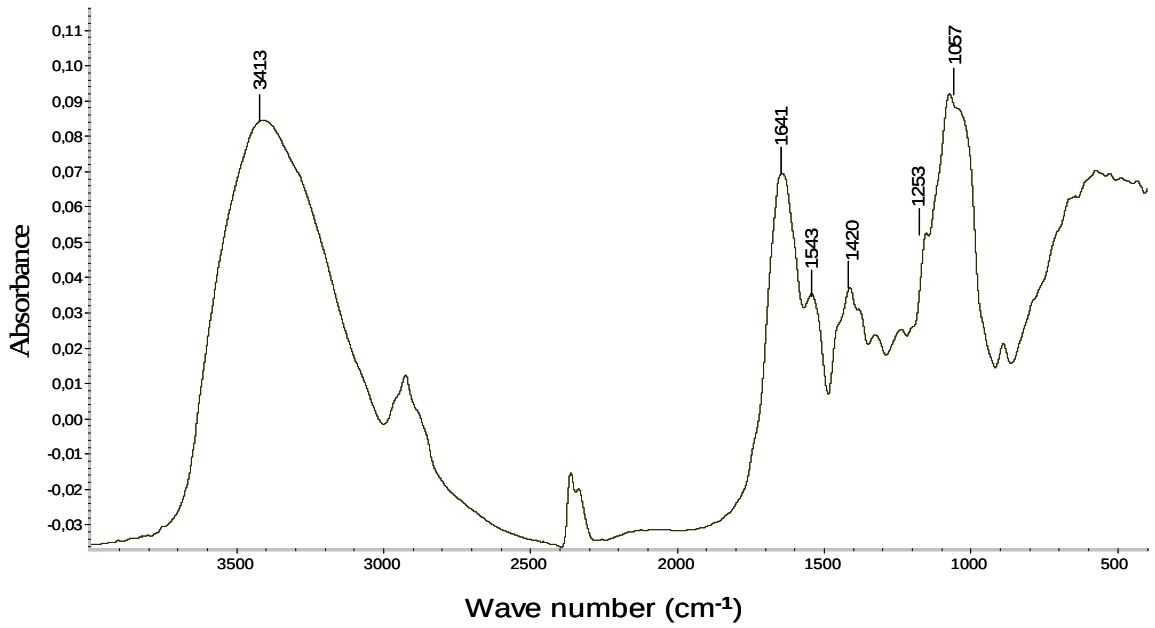

Fig. 3 Fourier transform-infrared spectroscopy spectrum of EPS-1 produced by the strain MC3B-10.

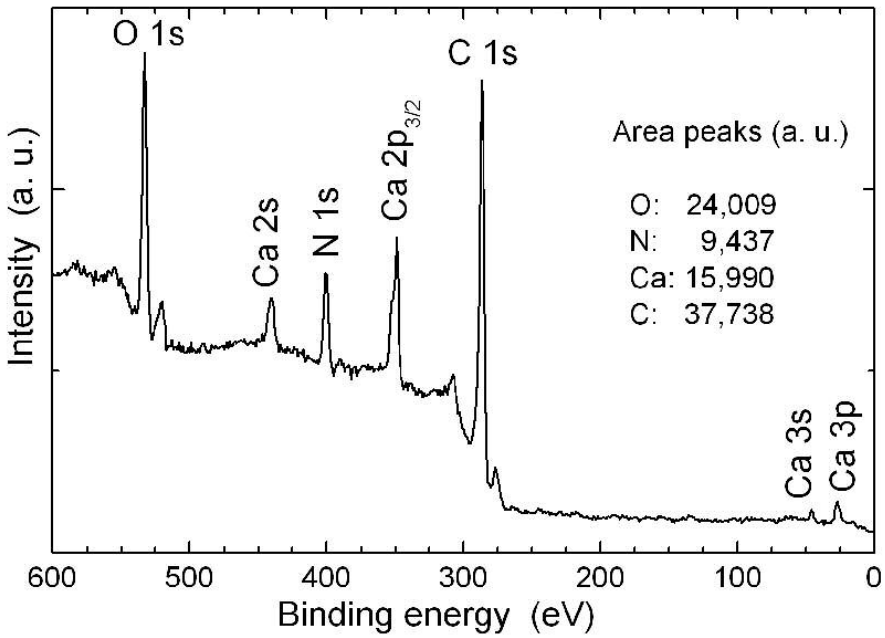

Fig. 4 XPS spectrum of the polymer produced by intertidal isolate MC3B-10.

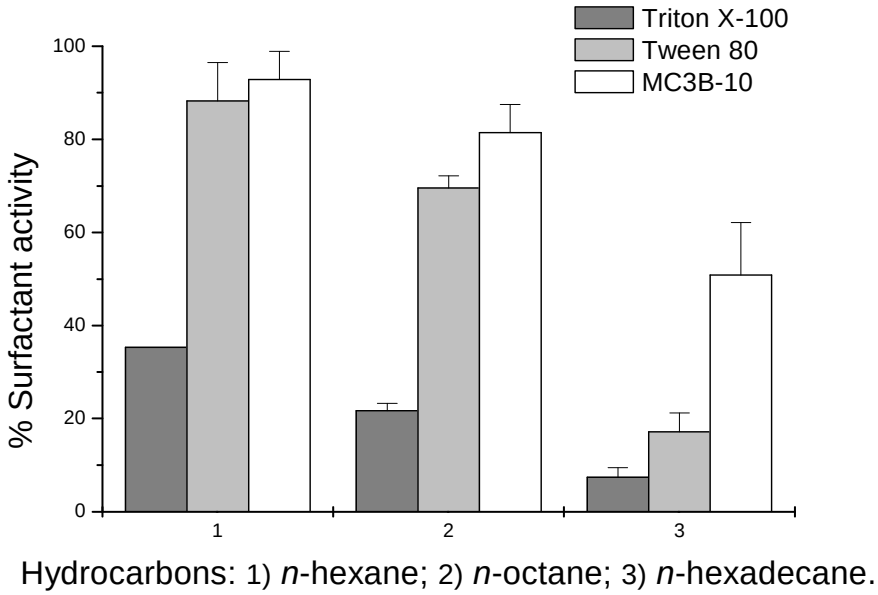

Fig. 5 Surfactant activity (emulsifying) of polymer MC3B-10 and commercial surfactants. Values are means of triplicate determinations ± S.D.