FEMS Microbiology Ecology 53 (2005) 157–166

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

An antarctic psychrotrophic bacterium *Halomonas* sp. ANT-3b, growing on *n*-hexadecane, produces a new emulsyfying glycolipid

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Received 17 June 2004; received in revised form 26 August 2004; accepted 1 September 2004

First published online 2 November 2004

Abstract

A bacterial strain ANT-3b was isolated at the sea-ice seawater interface from Terra Nova Bay station, Ross Sea, Antarctica. It was isolated on mineral medium supplemented with 2% diesel fuel as a sole carbon and energy source and grown routinely on 2% *n*-hexadecane. Analysis of 16S rRNA gene sequence indicates that the strain has 99.8% sequence similarity with *Halomonas neptunia*. The strain ANT-3b was grown in mineral medium supplemented with *n*-hexadecane between 4 and 20 °C, but not at 30 °C. The maximum degradation rate of the *n*-alkane was measured at 15 °C, with 5.6 ± 1.7 mg O_2 μg^{-1} protein d^{-1}. The strain ANT-3b produced emulsifying compounds when grown on *n*-hexadecane, but not on mineral medium supplemented with D-fructose. A preliminary characterisation of the emulsifier was carried out. The lipid moiety contained a mixture of fatty acids with a following composition in molar ratio: caprylic acid 18.85, myristic acid 1.0, palmitic acid 9.68, palmitoleic acid 5.69 and oleic acid 1.26. The polysaccharide moiety also contained a mixture of sugars with the following molar ratio: mannose 1.71, galactose 1.00 and glucose 2.96. The molecular weight of the glycolipid component determined by gel permeation chromatography was in the 18 kDa range and contained smaller fragments, possibly oligomeric contaminants. Transmission electron microscopy showed contact between the glycolipid secreted by the strain and *n*-hexadecane broken down to nanodroplets at the water interface, to form a material with mesophase (liquid crystal) organisation.
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Keywords: Cold-adapted; *n*-Hexadecane oxidation; Fatty acids; Emulsifier; Gel permeation

1. Introduction

Aliphatic and aromatic hydrocarbons of petroleum origin are serious environmental pollutants due to their persistence and high toxicity to all biological systems. Oil pollution of the oceans has been a problem ever since we began to use fossil fuels. Biodegradation by naturally occurring populations of microorganisms is a major mechanism of oil removal from the environment. Bacteria in particular are considered to be the best agents for the degradation of hydrocarbons originating from oil spills. Oil degradation rate is directly related to availability of hydrocarbons to microorganisms. For example, in *Pseudomonas aeruginosa*, *n*-hexadecane uptake is an energy-dependent process [1]. Alkane transport into microbial cells occurs by direct contact with larger alkane droplets and by pseudosolubilisation

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doi:10.1016/j.femsec.2004.09.013

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around cell envelopes. This process is often mediated by several emulsifying molecules, which facilitate uptake of hydrocarbons [1,2]. The production of different types of emulsifying and/or surface-active molecules facilitates the formation of hydrocarbon micro-micelles in water [3].

Most known biosurfactants and emulsifiers are complex molecules that consist of several lipidic functional groups linked to hydrophilic carbohydrate moieties with complexity ranging from simple disaccharides to branched oligosaccharides and polysaccharides. Many other potential emulsifier molecules can be identified at cellular level; however, these molecules are involved in precise biological functions and their use as anti-pollutant agents can hardly be foreseen. On the other hand, emulsifiers and biosurfactants produced by bacteria have converging functions suggesting that they are very important and that they developed in parallel with genotype and phenotype [4].

Research on microbial emulsifying agents has mainly been focused on mesophilic bacteria [4,5]. Few publications report on emulsifiers and surfactants produced by psychrophilic and psychrotrophic bacteria [6–10]. Because these molecules are produced at low temperatures, they are of great interest for environmental biotechnology, especially bioremediation. Bioavailability of hydrocarbons at low temperatures significantly decreases due to substrate solidification. Isolation of cold-adapted microorganisms, which produce specific molecules that increase emulsification of hydrocarbons, could have promising applications.

In this paper, we identify and describe a cold-adapted bacterial strain designated as ANT-3b that was isolated from Antarctic seawater and grown in medium culture enriched with diesel fuel as sole carbon source, and grown routinely on *n*-hexadecane. The aim was to determine some molecular characteristics of the emulsifier visibly produced at 15 °C. Physiological characteristics, isolation and compositional analysis of this complex new surface-active compound are also reported.

2. Materials and methods

2.1. Sampling

Superficial seawater samples were collected at Camp B site 3, Ross Sea, Antarctica (74°42′00″S, 164°06′89″E), during an expedition in summer 1999–2000. A 40-cm hole was drilled in the pack. Water samples were collected aseptically at the water–ice interface in Niskin bottles sterilised by washing with a solution of 1 N HNO_3. Samples were processed immediately in the laboratory of the Italian Base Terra Nova Bay in Antarctica.

2.2. Culture conditions and isolation

Seawater was incubated at 4 °C in the presence of 2% v/v diesel fuel until a significant cell density was observed in the flask. The diesel fuel (Esso Italiana) for diesel engine vehicles is composed of a mixture of *n*-alkanes (C_{12}–C_{28}) with traces of aromatics (<30 ppm polycyclic aromatic hydrocarbons (PAH)) and less than 1% total additives; it has a density of 0.830 g cm^{-3} at 15 °C and a viscosity of 2.0–4.5 mm^2 s^{-1} at 40 °C. The diesel fuel was filtered through a 0.2-μm pore size Teflon filter (Sartoriuos) for sterilisation and particle removal. Bacterial suspensions were transferred to the mineral medium (MM) containing: 24.0 g NaCl, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.7 g KCl, 2.0 g KH_2PO_4, 3.0 g Na_2HPO_4, 1.0 g NH_4NO_3 per litre of distilled water and incubated again under the same conditions for three weeks. Diesel fuel was the sole carbon and energy source. Colonies appearing on plates were purified by streaking at least three times on solid mineral medium spread with 50 μl diesel fuel.

Growth was suspended in 1.5 ml fresh mineral medium and 30% sterile glycerol and stored in several cryo-vials at −80 °C. A few other bacteria from different colonies were grown on mineral medium amended with diesel fuel, but only one strain thrived in this medium. This isolate was Gram-negative, aerobic organism identified as strain ANT-3b. It was grown routinely on 2% *n*-hexadecane, as sole carbon and energy source, replacing diesel fuel in mineral medium.

2.3. Growth on n-hexadecane

Strain ANT-3b was inoculated 1:100 (3.2 ± 1.2 μg protein ml^{-1}) in 200 ml flasks containing 50 ml mineral medium amended with 2.0% *n*-hexadecane. Each flask was incubated in static mode at either 4, 15 or 30 °C in different incubators. Growth was monitored by determination of protein content [11] as a measure of bacterial biomass.

2.4. Respirometric test

Tests were performed with pure liquid culture. Strain ANT-3b was inoculated in 50 ml mineral medium amended with *n*-hexadecane (2%) as sole carbon and energy source and incubated for 12 days at 15 °C while stirring. Oxygen consumption by strain ANT-3b was used to determine *n*-hexadecane degradation indirectly. It was measured with automated OxiTop® control system (WTW, Wissenschaftlich-Technische-Werkstaetten, Weilheim, Germany), in sealed sample vessels, with pressure sensors, maintained at a constant temperature. As biological processes consume oxygen and form carbon dioxide, this gas was captured by an alkaline absorbing agent (sodium hydroxide) in a glass side

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arm. At intervals of 20 min, decreased pressure in the headspace of the bioreactor was recorded by remote sensing as suggested by the protocol. Pressure was transformed into biological oxygen demand (BOD) using the equation in the OxyTop® protocol. At different times 3.0 ml aliquots were sampled to determine the protein content [11]. The only bioreactor allowed oxygen replacement in the headspace and growth was therefore limited by inorganic nutrients and carbon source. Oxygen consumption was calculated in relation to protein content.

2.5. Determination and analysis of 16S rRNA gene sequence

A single colony of strain ANT-3b, isolated on an agar plate, was harvested and dissolved in 50 μl of double-filtered distilled H_2O and kept at 100 °C for 5 min. Amplification of 16S rRNA gene and nucleotide sequencing were performed by Bact16S service Bio Molecular Research (CRIBI Biotechnology Centre, University of Padua, Italy). A complete 16S rRNA gene sequence was determined by amplification with an ABI 9600 thermocycler (Perkin–Elmer Applied Biosystems). Amplification product was purified with QIAquick PCR purification columns (Qiagen) following the manufacturer's protocol. Direct sequence determination of the purified DNA was carried out using a Prism Ready Reaction DyeDeoxy Terminator sequencing kit according to the protocols of the manufacturer (Perkin–Elmer Applied Biosystems) and an automated DNA sequencer model 3100 (Applied Biosystems). Analysis of the obtained sequences was performed using SIMILARITY_MATRIX version 1.1, SEQUENCE_MATCH version 2.7 and SEQUENCE_ALIGN version 1.7 from the ribosomal database project (RDP) and BLAST [12] as described elsewhere [10]. The NEIGHBOR program was used to construct phylogenetic trees from the evolutionary distance matrices by the Neighbor-joining method [13]. Random input order of sequences and multiple outgroup rooting with the 16S rRNA gene sequences of *Escherichia coli* and *Cellulomonas fermentans* were used to avoid potential bias introduced by the order of sequence addition. The resulting trees were analysed with the CONSENSE program to provide confidence estimates for phylogenetic tree topologies and to make a majority-rule consensus tree.

Sequence of 16S rRNA gene determined in this study was submitted to the EMBL nucleotide sequence database and assigned accession number AY616755.

2.6. Glycolipid extraction

Extraction of glycolipids was carried out from fraction H-2 following the procedure described below. After centrifuging the microbial broth (6000 rpm, 20 min, 20 °C), the supernatant was separated from the (underlying) aqueous fraction. The aqueous fraction was filtered, dialysed and freeze-dried (fraction H-1). The supernatant was extracted with methyl butyl ether (MtBE) and then centrifuged. The oil/water interface was collected, repeatedly centrifuged and washed with distilled water and then dried (fraction H-2). Preliminary analysis of protein content was carried out using the Bradford method [11]. Fraction H-2 was analysed by electrophoresis in SDS according to standard procedures.

2.7. Sugar composition of the glycolipid

The composition of the carbohydrate component in fraction H-2 was determined with hydrolysis in trifluoroacetic acid 2 N (100 °C, 6 h). Neutral monosaccharides released by hydrolysis were derivatised according to the procedure of Blakeney [14] and the alditol acetates formed were analysed by gas liquid chromatography (GLC) [7] in a GLC Perkin–Elmer Autosystem XL chromatograph using He as carrier gas. A capillary column SP2330, 30 m × 0.25 mm ID, 0.20 μm film (Supelco) was used. Column temperature was initially set at 200 °C for 1 min and programmed to rise linearly at 4 °C min^{-1} to 245 °C; the thermal program was halted isothermally at this temperature. Injector and detector block temperature was 280 °C. Quantification of analytes was performed by the internal standard method with inositol.

2.8. Fatty acid composition

Fatty acid composition of the lipid moiety was analysed by a method reported in the literature [15]. Sample H-2 (10 mg) was treated with 1.2 ml of 0.5 M methanolic NaOH at 70 °C for 1 h; then 1.5 ml 15% methanolic BF_3 was added and the mixture was incubated at 70 °C for 15 min. Methyl esters were extracted with 1.2 ml hexane. Phase separation was facilitated by addition of 1 ml saturated aqueous NaCl solution. Hexane extract was transferred to a vial, desiccated under dry N_2 flow, solubilised in 0.25 ml heptane and analysed with GLC. Instrument and operating conditions were as above, except for the thermal program (initial temperature 140 °C, initial hold 5 min, ramp 4 °C min^{-1} to 240 °C, hold for 10 min) and injector block temperature (250 °C).

2.9. Gel permeation chromatography (GPC)

Two glass columns (1500 × 18 mm) were packed with Biorad chromatographic materials: one column with Biogel P2, (fractionation range 100–1800 Da) and the other column with Biogel P-10 (fractionation range 1500–20,000 Da). A flow rate of 1.0 ml h^{-1} was delivered using a Pharmacia-LKB peristaltic pump. Column effluent was monitored for component peaks by using a

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Waters Differential refractometer R-401. In all GPC experiments the eluent contained NaCl 0.05 M, NaN_3 0.05% and SDS 0.05%.

Reference materials for calibration of the GPC system were sugar oligomers and dextran oligomers (Fluka) with suitable MW distribution range. Glucose was used as a marker of total volume (V_t) and dextran of Mr 500 kDa as a marker of excluded volume (V_o). Several samples of H-2 fraction were prepared by dissolving in the GPC eluent at a concentration of 6 mg ml^{-1}.

2.10. Neutral lipid localization by epifluorescence microscope

n-Hexadecane-induction of glycolipid was observed qualitatively in strain ANT-3b using the Nile Red protocol already tested [16] to determine lipopolysaccharide emulsan in *Acinetobacter* sp. RAG-1. The strain was grown at exponential phase in two different mineral media containing 1% fructose or 2% *n*-hexadecane as carbon and energy source. Cells of ANT-3b were grown five successive times in the mineral medium with fructose (1%) to suppress their ability to grow immediately on *n*-hexadecane. At the same time, other cells were transferred continuously to mineral medium amended with *n*-hexadecane as sole carbon source. A 1-ml aliquot of each culture in exponential phase was centrifuged at 12,000*g* for 5 min. The cell pellets were suspended in phosphate buffer saline (PBS) and incubated for 30 min. with 10 μl Nile Red (Nile Blue A oxazone) (Sigma), a fluorochrome specific for neutral lipids [16]. They were centrifuged and washed twice with 1 ml PBS at pH 6.9, containing 1.55 g K_2HPO_4 and 0.85 g $NaH_2PO_4 \cdot H_2O$ per litre. Finally, the cells were again suspended in PBS and 20 μl Nile Red (0.01 mg ml^{-1}) and then observed on glass slides with epifluorescence microscopy (Olympus mod. BX 51) equipped with digital camera. A control procedure was performed by adding fluorescein isothiocyanate (FITC) (Sigma), a general fluorochrome, to stain all cells. Original photos of specimens were exported in Tiff format to Power Macintosh G3. The relative fluorescence intensity was measured by NIH image 1.62b software.

2.11. Transmission electron microscopy observations

The cells grown on mineral media with fructose or *n*-hexadecane were prepared for transmission electron microscopy (TEM). After seven days of incubation at 15 °C, the cells of ANT-3b were harvested by centrifugation at 11,000*g*. The bacterial pellet was fixed for 1 h at 4 °C with 2.5% glutaraldehyde and 0.1 M lysine in 0.066 M cacodylate buffer, pH 7.2, for 30 min at room temperature. The cells were washed five times in the same buffer and post-fixed for 1 h at room temperature in 1% osmium tetroxide and then rinsed with distilled water, and embedded in Spurr resin. Ultrathin sections were prepared using a LKB II Nova Ultramicrotome with diamond knife. Sections were stained with 3.0% uranyl acetate solution for 15 min, washed once with distilled water and incubated in lead citrate for 10 min. TEM observations were performed with a JEOL JEM 100b (Tokyo, Japan) operating under standard conditions.

3. Results

Bacterial strain ANT-3b was isolated from seawater, sampled 700 m from the Italian Base in Antarctica in enrichment cultures with diesel fuel at 4 °C after 10 days of incubation. The strain was a Gram-negative, heterotrophic organism that produced round colonies on solid medium. It was cultivated routinely in liquid mineral medium with 2% *n*-hexadecane as sole carbon and energy source.

Strain ANT-3b was isolated at 4 °C and started growing on *n*-hexadecane at this temperature only after ten days, reaching a maximum of 15 μg protein ml^{-1} after 17 days, as an indirect measure of biomass (Fig. 1). The visible formation of extracellular products during growth caused problems in cell count under the microscope, even by the spread plate method in which colony forming units were underestimated due to natural cell aggregation. Biomass production was thus followed indirectly by determination of protein formation in the culture. Strain ANT-3b thrived at 15 °C in the presence of *n*-hexadecane as sole carbon and energy source. It started growing after one day and reached a maximum biomass (50 μg protein ml^{-1}) after seven days. Growth of ANT-3b was not detected after 15 days at an incubation temperature of 30 °C. However, growth was observed up to 21 °C.

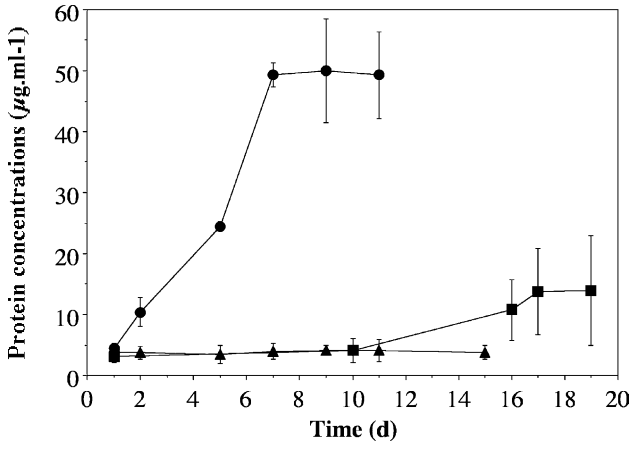

Fig. 1. Growth of *Halomonas* sp. ANT-3b determined as protein production (μg ml^{-1}) at 4 °C (■), at 15 °C (●) and at 30 °C (▲) in mineral medium supplemented with 2% *n*-hexadecane.

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An indirect test to determine *n*-hexadecane oxidation was performed by determining oxygen respiration using the Oxytop™ protocol at 15 °C. The biological oxygen demand (BOD) of strain ANT-3b started increasing after two days of incubation, one day after the start of growth, and reached a maximum of 1000 mg O_2 l^{-1} after 11 days. Hydrocarbon oxidation was calculated in relation to biomass formation with a rate of 5.6 ± 1.7 (mg O_2) (μg protein)$^{-1}$ d^{-1}. The measurements of BOD controls in the same conditions with dead cells and in the presence of live cells but absence of *n*-hexadecane were <0.5 mg O_2 l^{-1}.

The species of strain ANT-3b was finally determined by sequencing the gene encoding 16S rRNA. Phylogenetic analysis was performed on aligned sequences of the 16S rRNA gene using Maximum Parsimony (MP) and Maximum Likelihood (ML). An almost complete sequence of gene encoding 16S rRNA in ANT-3b isolate was determined (1471 bp). Preliminary RDP databases [12,17] indicated that the organism belongs to the gamma *Proteobacteria*. The sequence was manually aligned against representatives of gammaproteobacteria using the secondary structure model of bacterial 16S rRNA [18]. On the basis of 16S rRNA similarity, ANT-3b showed an apparent relationship with bacteria belonging to the *Halomonas* assemblage and formed a stable phyletic group within a heterogeneous cluster of *H. variabilis-neptunia-glaciei*, consisting mainly of strains isolated in cold environments (Fig. 2). The closest relatives of strain ANT-3b are *H. neptunia* DSM15720^T (98.6%) 16S rRNA gene sequence identity [19], *H. glaciei* JCM11692^T (99.1%), and *H. variabilis* DSM3051^T (98.6%) and several other not yet validly described halomonads isolated from polar seas (99.4–99.6%).

During the growth of *Halomonas* sp. ANT-3b on *n*-hexadecane at 15 °C a floating emulsion formed at the air-medium interface. The emulsifying agent was collected and purified (fraction H-2) for chemical and physico-chemical analysis of the glycolipid. The composition of carbohydrates in fraction H-2 was determined by

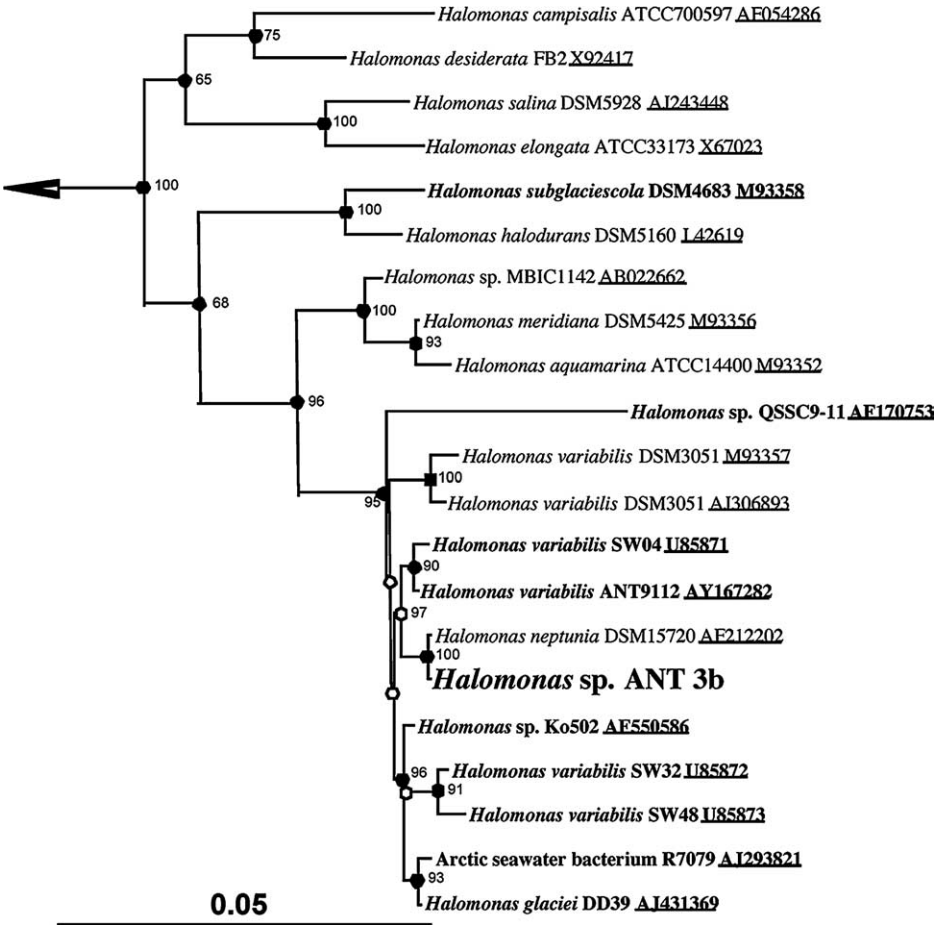

Fig. 2. Estimated phylogenetic position of *Halomonas* sp. ANT-3b among the closely related representatives of the Halomonadaceae family, as derived from 16S rRNA gene sequence comparisons. *E. coli* and *C. fermentans* were used for multiple outgrouping and rooting, respectively. Bacteria isolated from cold environments are in bold. Numbers at nodes, related to positioning of the sequences used, indicate percentage occurrence in 100 bootstrapped trees (only values >60 are shown). White circles show affiliations obtained by comparison of full sequences; black circles indicate positions confirmed by comparison of full and partial (33) sequences. Bar indicates a genetic distance of 0.05.

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GLC of the alditol acetate derivatives. Monosaccharide peaks were identified by calibration with standard monosaccharides. All peaks in the hexose range were clearly identified. Two peaks between the pentose and hexose ranges remained unidentified at this stage. Quantification of analytes was performed by the internal standard method with inositol, as internal standard, giving the following composition in molar ratio: Man = 1.71, Gal = 1.00, Glu = 2.96. Neither rhamnose nor aminosugars were present, nor could other common sugars be assigned to the unknown peaks.

Fatty acid composition of the lipid moiety was analysed using GC after derivatisation to methyl esters. Fatty acid peaks were identified with comparing retention times with those of the standards. Five of the eight peaks were unambiguously identified. Analytes were quantified by the internal standard method with sodium nonadecanoate giving the following composition in molar ratio: caprylic acid: 18.85, myristic acid: 1.00, palmitic acid: 9.68, palmitoleic acid: 5.69, oleic acid: 1.26. The approximate ratio of $C_{8:0}$, $C_{16:0}$, $C_{16:1}$ was therefore 3, 1.5, 1, for the major components, with minor amounts of other acyl chains.

Other attempts were made to characterise the H-2 fraction, the poor water solubility of which did not raise problems in chemical analysis where degradative methods were used. Although the solvent DMSO was found to dissolve the sample, preference was given to an aqueous solution containing 0.05% SDS as co-solute for the chromatographic (GPC) determination of molecular weight. This solvent was much more amenable than DMSO in the column and refractive index detector, because DMSO is hygroscopic. The lower limit of the molecular weight of the glycolipid component of fraction H-2 was determined to be around 18 kDa (Fig. 3), with other smaller fragments (in minor amounts) that could arise from degradation or oligomeric contaminants. Analysis of protein content in the H-2 fraction was checked by electrophoresis in the same system in SDS and confirmed the absence of protein bands. The molecular weight needs to be confirmed by more rigorous methods (e.g., mass spectrometry or light scattering), however, the value obtained indicates that the glycolipid with emulsifying activity has a molecular weight in the range of similar biopolymers found in certain Gram-negative microorganisms [20].

To determine whether extracellular production of this emulsifying agent by *Halomonas* sp. ANT-3b only occurrs in the presence of *n*-hexadecane or always, the cells were also cultured in mineral medium with D-fructose. The neutral lipid distribution on cells was performed using Nile Red, a specific fluorescence dye for neutral lipids. Fluorescence of this chromophore was only evident in the cells induced to oxidise *n*-hexadecane (Fig. 4(a)), whereas non-induced cells, transferred five times to D-fructose mineral medium, showed fluorescence reduced by 75%, as measured with imaging analysis (Fig. 4(b)). A control to this staining test was performed using fluoresceine isothiocyanate (Fig. 4(c) and (d)), with specimens cultured in *n*-hexadecane and fructose.

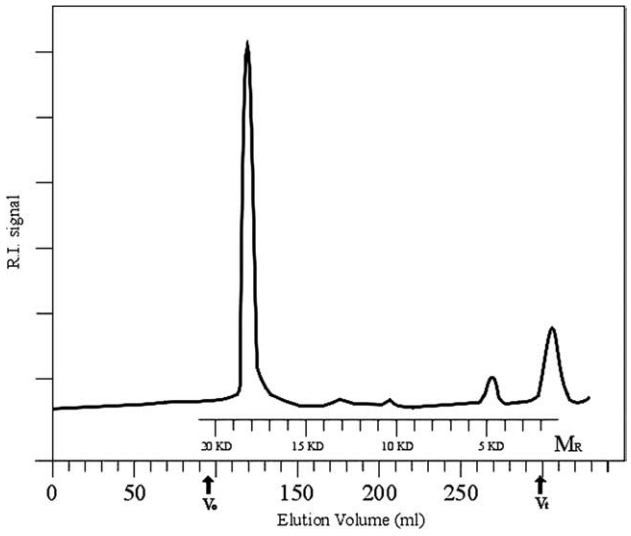

Fig. 3. Gel permeation chromatography of H-2 fraction on Biogel P-10 column (fractionation range: 1500–20,000 Da). The major peak is at 18 kDa.

Adhesion of strain ANT-3b to *n*-hexadecane was also investigated by optical microscope, observing the colonisation of oil droplets and disruption of the oil-water interface by the glycolipid (Fig. 5(a)). Morphology of *Halomonas* sp. ANT-3b cells growing on *n*-hexadecane was also determined by TEM analysis and compared with those growing on D-fructose, a carbon and energy source incapable of inducing the emulsifying agent. The cells growing on D-fructose were distinctly rod-shaped (Fig. 5(b)). On the contrary, cells growing on *n*-hexadecane had poorly defined morphology and a halo of extracellular material. The cells were so turgid as to resemble cocci (Fig. 5(c)). TEM showed also that the glycolipid was secreted abundantly in the medium and hydrocarbon droplets were clearly visible, suggesting a reduced surface tension of the oil droplets (Fig. 5(d)). On closer observation a contact area of composite material was evident between oil and the glycolipid (Fig. 5(e)). Some of this material was surrounded by a typical texture that can be attributed to the presence of mesophases [21].

4. Discussion

We isolated a bacterium that grows on n-alkane by virtue of a novel emulsifying agent that it produces. This ability is important for survival in cold environments where the bioavailability of hydrocarbons drops dra-

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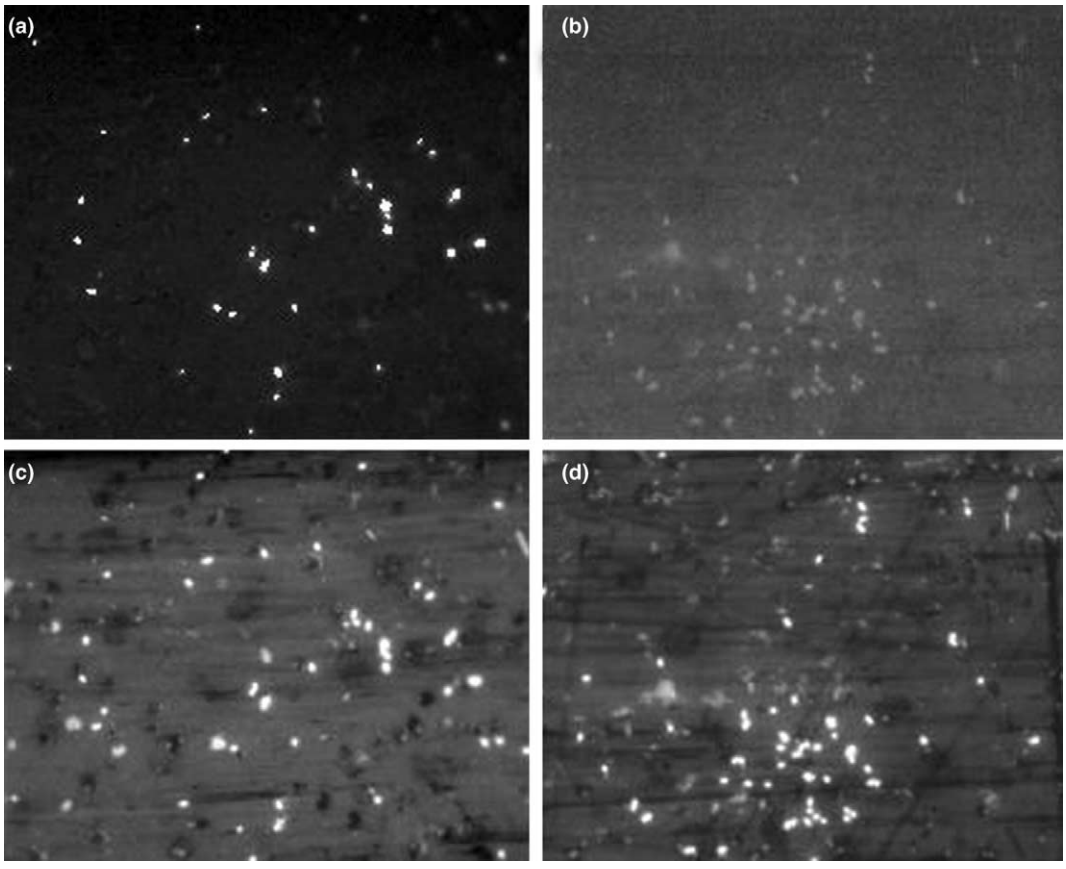

Fig. 4. Microscopical determination (×1000) of glycolipid formation in *Halomonas* sp. ANT-3b cells by means of Nile Red, a chromophore for neutral lipids, and its control with non-specific dye such as fluoresceine isothiocyanate. (a) Cells growing on mineral medium supplemented with *n*-hexadecane (2% w:v) and stained with Nile Red. (b) Cells growing on mineral medium supplemented with D-fructose (2% w:v) and stained with Nile Red. Note the reduced emission of fluorescence. (c) Control of the same cells growing on mineral medium supplemented with *n*-hexadecane (2% w:v) and stained with fluoresceine isothiocyanate. (d) Control of the same cells growing on mineral medium supplemented with D-fructose (2% w:v) and stained with fluoresceine isothiocyanate.

matically due to their solidification. Secretion of an emulsifier produced by a cold-adapted bacterium could also stimulate other microorganisms to feed on hydrocarbons at low temperature. Various gammaproteobacteria have the ability to produce biosurfactant molecules. For example, the genus *Pseudomonas* produces rhamnolipids, which have biosurfactants containing rhamnose and β-hydroxydecanoic acid [22,23]. It has been claimed that synthesis of these molecules is linked to alginate production [24]. Rhamnolipids are amphiphilic surface-active glycolipids usually secreted in the medium [25,26], and they could have applications in combating marine oil pollution, especially removing oil from sand [27].

Various other emulsifying molecules produced by gammaproteobacteria are the lipopolysaccharide, emulsan [28,29], and the glycoprotein alasan, [30,31], isolated from different *Acinetobacter* species. A new surfactant activity was recently found in another gammaproteobacterium *Alcanivorax borkumensis* [32]. It contains glycine and β-glucopyranoside esterified with 3-hydroxy-hexanoic, -octanoic and decanoic acids, but has a smaller molecular weight than that produced by strain ANT-3b. Unfortunately, the complicated molecular structure of the glycolipid has not yet been solved by NMR spectroscopy analysis.

Halomonas sp. ANT-3b is a cold-adapted gammaproteobacterium that produces an emulsifying glycolipid in the presence of *n*-hexadecane. Species determination was necessary to assess whether it was a new species or other known strains of the same species of Antarctica origin. The highest similarity (99.8%) was with *H. neptunia* sp. nov. isolated from deep-sea [19]. Common lineage of strain ANT-3b by phylogenetic analysis [33] with *H. neptunia* and Antarctic halomonads ANT9112 and SW04 was confirmed.

The branching point of strain ANT-3b was stable, as the corresponding bootstrap value was 100%. A very similar tree was constructed using the Jukes-Cantor algorithm (data not shown). However, strain ANT-3b differed significantly in physiological and biochemical properties from the novel species *H. neptunia* [19].

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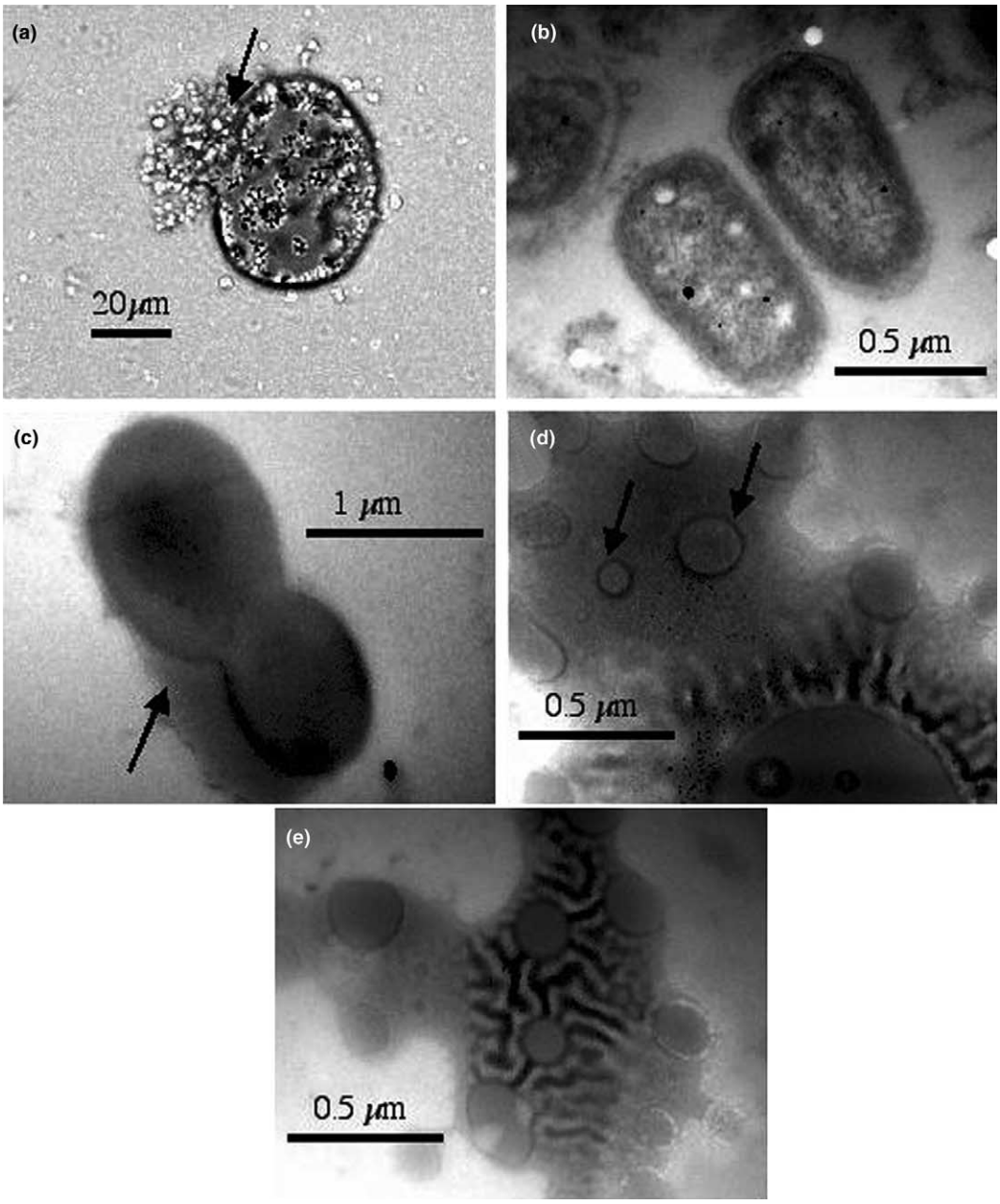

Fig. 5. (a) Light microscope image (transmission mode) of *Halomonas* sp. ANT-3b cells adhering to *n*-hexadecane droplet and breaking through the water-oil interface (arrow). (b) Transmission electron micrograph of *Halomonas* sp. ANT-3b cells growing in mineral medium supplemented with D-fructose. (c) Transmission electron micrograph of *Halomonas* sp. ANT-3b cells growing in mineral medium supplemented with *n*-hexadecane. Note turgid shape (resembling coccus) and extracellular halo (arrow). (d) Interaction of glycolipid with dispersed *n*-hexadecane droplets of different sizes (arrows). (e) Detail of glycolipid/*n*-hexadecane interaction forming quasi-ordered structure of mixed glycolipid and hexadecane.

The strain ANT-3b isolated from this area shows psychrotolerant behaviour, growing on *n*-hexadecane as sole carbon and energy source at 4 °C, with an optimum at 15 °C, while no growth was detected at 30 °C. Since it is known that under strictly aerobic conditions *n*-hexadecane is degraded by *n*-alkane monoxygenase, it has not been considered necessary to ascertain its presence. However, further studies are needed on the genes and enzymes of strain ANT-3b involved in aliphatic hydrocarbon degradation, even though only the *alk* operon is known so far to code for *n*-alkane degrading enzymes in strictly aerobic bacteria [34].

Chemical characterisation of the glycolipid revealed that it contains both saturated and unsaturated fatty acids of different chain length [35,36]. Caprylic acid ($C_{8:0}$) appears to be the most abundant, followed by palmitic ($C_{16:0}$) and palmitoleic ($C_{16:1}$) acids. The presence of short-chain fatty acids, such as caprylic, is rarely re-

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ported in glycolipids [32]. The variability of fatty acid length is presumably related to the peculiar ability of *Halomonas* sp. ANT-3b to emulsify *n*-hexadecane at low temperatures, but it could also be derived from the presence of several glycolipids. Glycolipids with regular long chain fatty acids presumably undergo lipid phase transition more readily, losing their emulsifying ability. Although the molecular structure of the whole glycolipid has not yet been determined, its composition and functions raise several questions about adaptation of this strain to environmental conditions of low temperature and oil feedstock. The peculiar chain length could possibly protect integrity and function of cell membrane [37]. The oil-glycolipid contact at the interface between the organic and aqueous phases, have liquid-crystalline birefringent patterns [21]. The temperature stability of the mobile lipid phase is also of interest in view of the peculiar asymmetric composition of the glycolipid component. The interference pattern calls for in vitro microscopic and diffractometric analysis of the texture of the mixed-lipid phase with the aim of characterising these mesophasic structures [21].

A comment seems necessary about the macromolecular characteristics of the glycolipid. Its molecular weight, while requiring confirmation by more rigorous methods of molecular mass determination (e.g., light scattering), indicates that the glycolipid is in the 10^4 Dalton range. This is quite different from sizes of most known surfactant glycolipids [38], which contain either disaccharides (rhamnose, trehalose, cellobiose) or polysaccharides such as emulsan or liposan [5]. The dimensions of the glycolipid from *Halomonas* sp. ANT-3b are rather similar to those of some O-antigenic polysaccharides associated with the LPS core of Gram-negative microorganisms [20]. Although sugar composition indicates some similarity with the lipo-oligosaccharide fraction of psychrophilic *Pseudoalteromonas haloplanktis* TAC 125 [7,8], the molecular weight of the two lipo-oligosaccharides (2134 and 2332) is clearly much smaller than determined here. One may infer that eight to ten of such units are linked in what can be considered a synergic emulsifier.

A possible presence of both lipo-oligosaccharides and lipo-polysaccharides of different molecular weight but perhaps similar composition seems to be inferred in the case of *Pseudoalteromonas haloplanktis* TAC 125 [7]. It could be important to know whether the production of these macromolecules follows distinct metabolic lines or arises from differentiation in extracellular post-synthesis polymerization. Similar problems were indeed encountered in the study of polysaccharides from the genera *Rhizobium* and *Agrobacterium*, leading Zevenhuizen [39] to propose a common route for the biosynthesis of two different polysaccharides produced in connection with different environmental conditions.

Acknowledgements

This research was financed by PNRA project 1999–2003 (Progetto Nazionale Ricerche in Antartide). The authors thank Michail Yakimov for the phylogenetic tree and suggestions for the manuscript. They also thank to Claudia Faleri for TEM analysis.

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