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Characterization of a hydrocarbon degrading psychrotrophic Antarctic bacterium

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Abstract: Antarctic soil chronically exposed to gas-oil was analysed in order to isolate and study the growth conditions of hydrocarbon degrading bacteria. Soil samples taken near the shoreline in Jubany Station (King George Island, South Shetland Islands) were used as inoculum in liquid culture media with crude oil as sole carbon source. A psychrotrophic *Acinetobacter* strain was isolated and selected for further investigations. Effects were studied of temperature, initial pH, NaCl concentration and different chemical structure of the hydrocarbon on growth. Degradation rate was determined with n-dodecane and n-hexadecane. Growth of *Acinetobacter* ADH-1 showed no differences at an initial pH of 7.0, 7.5 and 8.0. Optimum temperature ranged between 25–30°C but the strain was capable of growing on n-dodecane at 4°C. Growth was observed in the presence of 3.5% NaCl. A decrease in the surface tension values was observed in the culture broth during the first 20 h of incubation (from 68 din cm^{-1} to 31 din cm^{-1}). This proved to be related to the cellular fraction of the culture. The study shows that *Acinetobacter* ADH-1 is a psychrotrophic bacteria able to grow with hydrocarbons as sole carbon and energy source and could be potentially useful to design bioremediation processes in temperate and cold climate areas.

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Key words: Antarctic bacteria, biodegradation, hydrocarbons, psychrotrophs

Introduction

In Antarctica, the permanently low temperatures and the limited previous exposure to anthropogenic hydrocarbons may suggest a low rate of response by the indigenous microflora when exposed to a carbon source of oil or its derivatives (Delille & Vaillant 1990, Kennicutt 1990). The continuing human presence in this area generates a potential risk of pollution through the activity of the scientific stations, fishing activities and tourist ships (Platt *et al.* 1981). The wreck of the *Bahia Paraiso* on Anvers Island in 1989 is a recent example of marine pollution by hydrocarbons whilst the presence of natural hydrocarbon beds in the Antarctic seas (MacDonald *et al.* 1988) may also be a source of localised oil leakage.

Pollutants may be removed from the environment in a variety of physical, chemical and biological ways. Bacterial attack can be an important process in some environments and specific strains have been investigated to asses their capabilities to degrade particular compounds. Today, bacterial bioremediation techniques offer new possibilities to accelerate the pollutant degradation, either by adding the appropriate nutrients that are required by the naturally occurring microorganisms (Mills & Frankenberger Jr. 1994, Rivet & Mille 1993) or, when the bacterial populations are not adequate, by seeding pure or mixed cultures of bacteria having the metabolic pathways that permit a faster metabolization of the contaminant (Liu & Suflita 1993). In order to develop the bioremediation techniques, a knowledge of the taxonomic and physiological characteristics of the bacterial strains potentially useful in these processes is essential. In this paper we describe the isolation and characterization of a hydrocarbon degrading bacterium from an Antarctic soil chronically exposed to gas-oil. The restrictions on the introduction of alien organisms into the Antarctic environment make it essential to establish the bioremediation potential inherent in the native species.

Materials and methods

Study area

Samples were collected in sterile flasks from soils around the gas-oil storage tanks of the Jubany Scientific Station (62°14'S, 58°40'W) on King George Island (South Shetland Islands) during the 1988–89 Argentine Antarctic Research Expedition. The tanks area is located near the shoreline and soil had been exposed to small spills for many years during fuelling operations. The soil samples were kept at 4°C.

Enrichment cultures, isolation and identification of the strains

Liquid cultures were made in 300 ml Erlenmeyer flasks with 60 ml of sterile saline basal medium (SBM) containing ($g\ l^{-1}$):

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NH_4NO_3 4, Na_2HPO_4 8.9, KH_2PO_4 1.8, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.01, $FeSO_4.7H_2O$ 0.01, EDTA 0.01, $CuSO_4.5H_2O$ 0.03, $ZnCl_2$ 0.01 at pH 7.5. For the enrichment procedure 1% v/v of crude oil (provided by YPF Oil Company) was added to SBM as sole carbon and energy source. Crude oil was used in the enrichment step to offer a complex mixture of hydrocarbons able to support the growth of degrading bacteria with different requirements. Suspensions of soil samples (0.1 g ml^{-1}) vigorously shaken were used as inoculum (1% v/v) and the cultures were incubated on a rotary shaker at 250 rpm and 20°C for 72 h. Four consecutive subcultures were made under the same conditions using 0.6 ml aliquots of the previous culture. After 120 h serial dilutions of the last culture were plated on a non-selective nutrient agar and incubated at 20°C in order to recover all the strains capable of growing in the crude oil medium. Colonies obtained were isolated and purified by re-streaking twice. The selection of the strain (*Acinetobacter* ADH-1) was based on growth of the isolated strains in aerated submerged culture with SBM and n-hexadecane (2%) as sole carbon and energy source under the same conditions as described above. In addition, crude oil degrading capacity was tested on microtitre plates with the Hanson *et al.* (1993) technique based on the colour change of the electron acceptor 2,6-dichlorophenol indophenol (DCPIP). All isolates were tested for morphology and mobility (phase contrast microscope), oxidase and catalase production, Gram reaction, Hugh-Leifson O-F test (Hugh & Leifson 1953), pigment production and spore formation. Flagella insertion was examined in all mobile strains. Gram-negative strains were identified at genus level using the scheme of Shewan (1971). In addition, the selected strain was identified at species level with the Rapid NFT (non-fermenters) kit of API System, MINITEK System and additional biochemical tests.

Physical and chemical factors affecting the growth of Acinetobacter *ADH-1*

The effect of initial pH, incubation temperature, NaCl concentration, chemical structure and composition of the hydrocarbonated substrate on growth of *Acinetobacter* ADH-1 was analysed in liquid culture (60 ml of SBM in 300 ml Erlenmeyers flasks shaken at 250 rpm). Pure and complex mixtures of hydrocarbons were analysed as growth substrates: n-hexadecane, n-dodecane, n-heptane, n-hexane, cyclohexane, pyrene, xylene, crude oil, aromatic gas-oil, hydrogenate gas-oil and kerosene. In all cultures hydrocarbon concentration was 2% except with pyrene and xylene where 0.1% was used. Duplicate flasks of n-hexadecane and n-dodecane cultures, and a sterile control were collected at different times and extracted twice with dichloromethane (10 ml). Hydrocarbon concentration was measured in these extracts using a Konik 3000 gas chromatograph equipped with a 30 m x 0.538 mm i.d. DB-1 fused silica capillary column (3.0 μm film thickness) with a flame ionization detector and nitrogen as carrier gas. Biomass was estimated by a modification of the Hug & Fiechter (1973) technique: after two consecutive extractions of the hydrocarbon with a solvents mixture (ethanol-chloroform-buthanol, 10:1:10, and a ratio of medium volume to solvent volume of 6:1) biomass was evaluated by dry weight (triplicate samples, 24 h at 105°C). This procedure was necessary because ADH-1 showed a heterogeneous growth in liquid media forming macroscopic complexes in association with the hydrocarbon drops due to the high cell surface hydrophobicity.

Surface tension of the culture medium during the growth was measured in triplicate with a Fisher surface tensiometer (Model 20). To determine whether the selected strain harboured plasmids, DNA was isolated by the Birnboim & Doly (1979) procedure and then separated by agarose gel electrophoresis

Results

From the enrichment cultures ten strains were obtained. Taxonomic analysis at genus level showed seven *Pseudomonas* spp, two *Acinetobacter* spp and one *Flavobacterium* sp. On the basis of growth capacity on SBM with n-hexadecane as carbon source, one *Acinetobacter* strain (ADH-1) was selected for further studies. Degradation capability of the strain was confirmed by the colour change of DCPIP from blue to colourless 12 h after inoculation. The physiological and nutritional characteristics of the ADH-1 strain are listed in Table I. Based on the results obtained, ADH-1 was classified as *Acinetobacter lwoffi*.

The effect of the initial pH of the culture medium (range between 6.0–8.0) on the growth of ADH-1 with n-hexadecane is shown in Fig. 1. Significant differences were not observed at initial pH values of 7.0, 7.5 and 8.0 either in the biomass

Table I. Biochemical and nutritional characteristics of the Antarctic strain *Acinetobacter* ADH-1.

Gram reaction	-	Methyl red test	-
Motility	-	Indol test	-
Spore formation	-	SH_2 production	-
Fluorescent pigment	-	Voges Proskauer reaction	-
Growth at 4°C	+	Nitrate reduction	-
Aesculin hydrolysis	-	Utilization of:	
Starch hydrolysis	-	D-glucose(aerob.)	+
Gelatin hydrolysis	-	D-glucose(anaerob.)	-
Growth in 2.5% NaCl	+	D-mannose	+
Growth in tween 80	+	D-xylose	-
Growth at pH 5.6	+	D-arabinose	+
Growth in Mc Conkey	-	Maltose	-
Production of:		Sacarose	-
Oxidase	-	N-acetyl D-glucosamine	+
Catalase	+	Mannitol	+
Arginine dehydrolase	-	Acetate	+
Lysine decarboxilase	-	Adipate	-
Ornitine decarboxilase	-	Caprate	-
b-Galactosidase(PNPG)	-	Citrate	+
Urease	+	Gluconate	+
Tryptophanase	-	Malate	+
Phenylalanine deaminase	-	Phenyl-acetate	-

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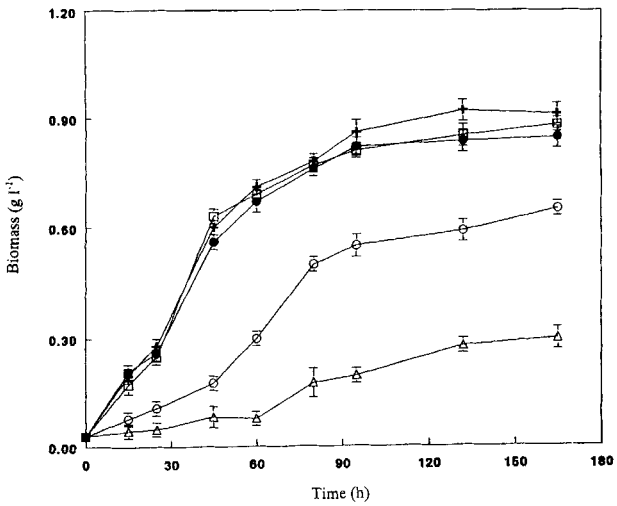


Fig. 1. Effect of initial pH on the growth (mean ± SD) of ADH-1 strain at 20°C with n-hexadecane as carbon source: Δ : pH 6.0, o : pH 6.5, ● : pH 7.0, +: pH 7.5, ❑ : pH 8.0.

production or in the specific growth rate (μ), whose values were *c.* 0.04 h^{-1}. At an initial pH of 6.5 the growth was substantially lower than at higher pH values, and at pH 6.0 the biomass was three fold lower than in the neutral or alkaline pH range.

Sodium chloride concentration can exert a strong influence on the growth and degradation capability of the bacterial strains, and knowledge of the optimum values is essential if any application to bioremediation in marine or high saline environments is being considered. Figure 2 shows the growth of ADH-1 at different NaCl concentrations on n-hexadecane. While the best growth was observed at low NaCl concentration, values as high as 3.5% w/v permitted a high biomass formation (1.3 g l^{-1} after 165 h). No appreciable growth was observed at 5% w/v NaCl. The total salt concentration of the SBM must be added to the NaCl concentration in the culture media. Thus, ADH-1 is capable of growing at high osmolarity values.

Temperature had a marked effect on the growth of ADH-1 (Fig. 3). At 25°C and 30°C no growth differences were observed with n-dodecane as carbon source, maximal biomass ranged between 1.74 g l^{-1} (at 25°C) and 1.84 g l^{-1} (at 30°C) and μ was similar at both temperatures (0.030 h^{-1} and 0.031 h^{-1} respectively). At 20°C, the biomass as well as μ showed lightly lower values (1.50 g l^{-1} and 0.028 h^{-1} respectively). At 5°C and 10°C a lag period was observed, which was longer at 5°C. The biomass achieved at the end of the analysis period (165 h) was similar at both temperatures (0.75 g l^{-1} at 5°C and 0.80 g l^{-1} at 10°C) and showed much lower values than those observed at the other culture temperatures studied. Growth of ADH-1 at different temperatures is shown with n-dodecane as carbon source because this n-alkane has a melting point (m.p.) of -9.6°C which permits incubation of cultures at any temperature above 0°C whereas n-hexadecane, with a m.p. of 18.2°C did not permit us to compare the growth at low and moderate temperatures. At any rate at 20°C, as can be observed in Fig. 4, the growth as well as the hydrocarbon consumption were very similar with n-dodecane and n-hexadecane as sole carbon sources and only a slight and not significant difference was observed in the substrate consumption rate during the growth phase. At the end of the study (165 h), at 20°C only 16 % of the initial n-hexadecane and 11% of the initial n-dodecane remained undegraded. Abiotic controls under assay conditions showed no changes in the hydrocarbon concentration. In both cases, despite the extent of hydrocarbon metabolization, the observed growth yield (Yx/s), calculated from biomass production (Δx) and hydrocarbon consumption (Δs) by the equation Yx/s = Δx/Δs,

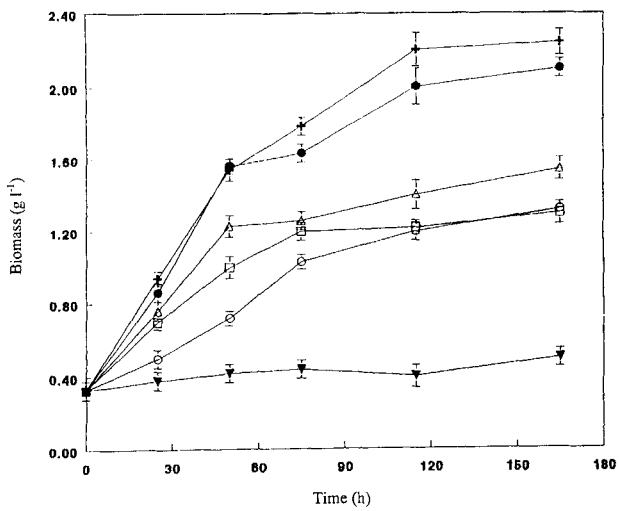


Fig. 2. Growth (mean ± SD) of ADH-1 strain at 20°C with n-hexadecane as carbon source and different NaCl concentrations. ●: 0%, +: 0.5%, Δ: 1.0%, ❑: 2.0%, O: 3.5%, ▼: 5.0%.

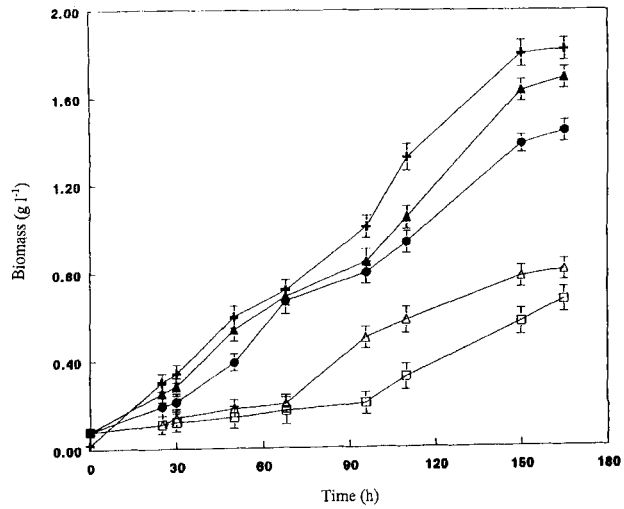


Fig. 3. Effect of culture temperature on growth (mean ± SD) of ADH-1 strain with n-dodecane as sole carbon source. ❑ : 5°C, Δ : 10°C, ● : 20°C, ▲ :25°C, + : 30°C.

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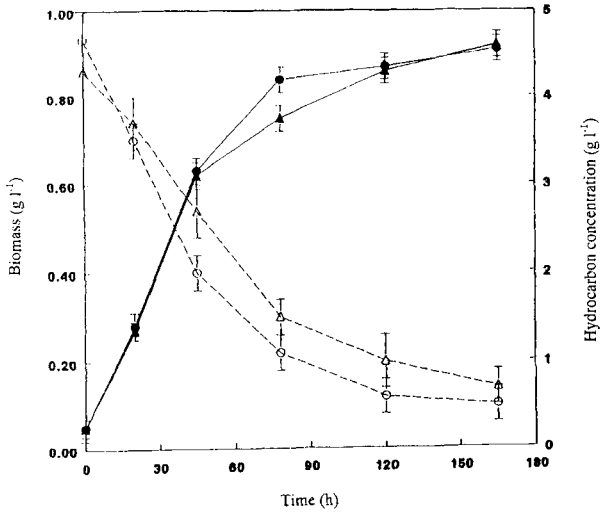


Fig. 4. Growth (mean ± SD) and hydrocarbon consumption of ADH-1 strain with n-dodecane (O) or n-hexadecane (Δ) as sole carbon source at 20°C. Closed symbols show biomass evolution, open symbols show the hydrocarbon concentration in the culture media.

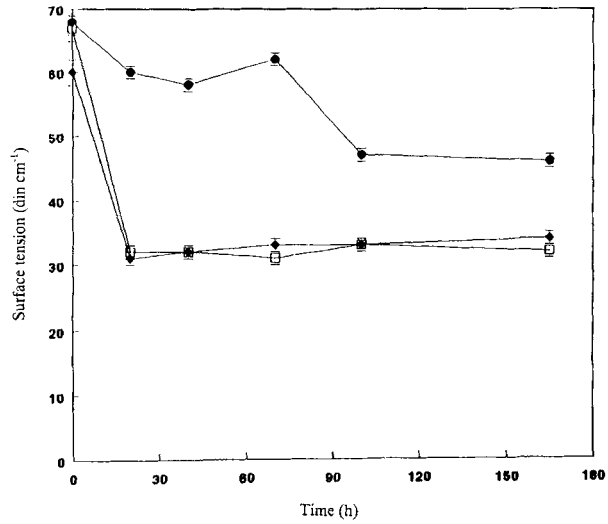


Fig. 5. Surface tension values (mean ± SD) in ADH-1 cultures. ◆ : values obtained from the total broth containing n-hexadecane. ● : values from the cell free supernatant. ❑ : values from cellular fraction resuspended in distilled water.

never reached values higher than 0.3 (Yx/s from n-hexadecane at 96 h was 0.28 and from n-dodecane at 78 h was 0.21) suggesting that an important fraction of the chemical energy of the hydrocarbon molecules was either consumed in other metabolic activities than biomass synthesis, or remained in the culture as only partially degraded metabolites. This comment is based on the knowledge of the high growth yield values that can be achieved by some microorganisms growing on highly reduced substrates such as hydrocarbons (Wagner *et al.* 1969).

Strain ADH-1 grew heterogeneously and a macroscopic cellular arrangement in association with hydrocarbons could be observed in liquid culture. In order to investigate the production of surface-active compounds, we analysed the evolution of surface tension (S.T.) in cultures of ADH-1 with n-hexadecane (Fig. 5). The culture broth showed a rapid decrease in S.T. (31 din cm^{-1}) at early stages of the cultivation and maintained these low values throughout the analysis period. When the broth was divided into a cell free supernatant and a cellular fraction that was resuspended in water, surface activity was associated principally with the cellular fraction (Fig. 5), which showed very similar S.T. values to those from the culture broth. In order to minimize the possible non specific effect of the cell particles on the S.T., biomass of other bacterial strains was resuspended in water and checked for changes in S.T. In all cases, the cellular effect of this control was small and did not justify the decrease in the S.T. values of the equivalent ADH-1 suspensions.

We have found (Table II) that ADH-1 grows not only on middle chain-length alkanes, but also on complex mixtures of crude oil, hydrogenated gas-oil, aromatic gas-oil and kerosene. A preliminary analysis showed no ability to grow on aromatic hydrocarbons such as pyrene and xylene. In addition, no appreciable growth was observed on short chain alkanes (octane and shorter). Finally, no plasmids were found in this strain by agarose 0.7% gel electrophoresis, either in cells cultured in the presence of hydrocarbons or in cells cultured in nutrient broth.

Discussion

Antarctica represents an extreme environment. There, the low temperature makes any microbial metabolic activity extremely slow when compared with temperate climates. Probably no success in the bioremediation process could be achieved using non-psychrotrophic microorganisms in this environment. Human activity represents a potential risk of pollution. In the marine environment, there are a number of different activities that contribute to the total hydrocarbon input, as has been well summarized by Cripps & Priddle

Table II. Growth of *Acinetobacter* ADH-1 on pure or complex mixtures of hydrocarbons as sole carbon source. In all cases hydrocarbon concentration was 2% except with pyrene and xylene where 0.1% was used.

Hydrocarbon	Growth
crude oil	+
aromatic gas-oil	+
hydrogenated gas-oil	+
kerosene	+
n-hexadecane	+
n-dodecane	+
n-hexane	-
n-heptane	-
n-octane	-
cyclohexane	+
pyrene	-
xylene	-

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(1991). In soils, spillage from fuel storage tanks seems to be the main source of hydrocarbons. It is clear that higher hydrocarbons content in soils, sediments and organisms occurs near the stations, and decreases away from these areas (Cripps & Priddle 1991, Cripps & Priddle 1995, Green & Nichols 1995).

ADH-1 is a psychrotrophic strain of *Acinetobacter* isolated from chronically contaminated Antarctic soil that proved to grow on hydrocarbons as sole carbon and energy source. The genera of hydrocarbon degraders found from the samples analysed agree with previous papers which indicated the predominance of gram negative bacteria in several different Antarctic habitats (Mac Cormack & Fraile 1990, Delille 1993, Vazquez *et al.* 1995), and *Pseudomonas* and *Acinetobacter* are probably two of the most abundant genera present in any hydrocarbon contaminated site (Ridgeway *et al.* 1990, Gutnick *et al.* 1991).

Results showed that strain ADH-1 grows efficiently on n-hexadecane at pH values between 7.0 and 8.0. Although, in general, *Acinetobacter* species have a slightly acid optimum growth pH (Towner *et al.* 1991), the higher optimum value observed could reflect a habitat adaptation (strain ADH-1 was isolated from the marine coast of Potter Cove) and, in fact, the pH of the soil in this area was 7.2 when measured according to the Seppelt (1992) procedure. This range of pH tolerance, in addition to the high salt resistance (Fig. 4), make the strain potentially adequate to decontaminate marine and coastal polluted sites. According to the definition of Morita (1975) ADH-1 is a psychrotrophic (and not a psychrophilic) microorganism with an optimum growth temperature above 20°C but capable of growing at near 0°C (Fig.2). This is not surprising because, as has been reported previously (Delille & Perret 1989), a large part of the coastal Antarctic bacterial community appear to be psychrotrophic rather than obligately psychrophilic. Taking this into account, we undertook the isolation at 20°C to obtain a higher number of isolates potentially useful in supporting a biomass production at moderate temperature (for the inoculum preparation) and compatible with the fermentation plants actually in use.

Although biomass achieved high values at optimum culture temperature (1.84 g l^{-1} at 30°C), the specific growth rate (μ) was low, even compared with other hydrocarbon degrading strains (Hutchinson & Campbell 1988, Espeche *et al.* 1994). The reason for this low μ could be related to the agglutinated growth shown by ADH-1 due to its adherence to hydrocarbon droplets. With this type of growth, where no homogeneous distribution of substrate and cells is possible, nutrient limiting conditions (carbon source, oxygen, etc.) could be present in one or many of the concentric cell layers formed around the insoluble carbon source, leading to a growth rate inhibition. Although this feature would determine a decrease in the efficiency of hydrocarbon consumption, the high affinity between the ADH-1 cells and the hydrophobic compounds represents an important advantage in order to initiate and maintain a strong contact between cells and hydrocarbons that, in fact, reduce the undesirable cell dispersion if this strain is used in a polluted natural environment. Also, the cell-hydrocarbon affinity could be caused by a cell-associated surfactant. This could explain the decrease in surface tension of the culture and the bigger effect of the cell fraction in comparison with that of the cell free supernatant. These results (Fig. 5) are in agreement with Neufeld & Zajic (1984) who observed a decrease in the surface tension by n-hexadecane grown cells themselves.

The incapacity to degrade short chain length alkanes seems to be a common feature in hydrocarbon degrader strains of *Acinetobacter* and only a few strains have been reported able to grow on decane or shorter chain length alkanes (Asperger & Kleber 1991). In all cases, this capacity was found to be related to the presence of an alkane-inducible cytochrome P-450 (Asperger *et al.* 1981). As occurs with ADH-1, only some of the total hydrocarbons from a complex mixture can be degraded by any particular strain. Hence, several Antarctic strains will have to be studied and a microbial consortia should be designed in order to make the microorganisms mediated hydrocarbon remotion more efficient, as remarked by other authors (Sasek *et al.* 1993, Ferrari *et al.* 1994).

No plasmids were detected in ADH-1 strain, which is not surprising since as far as we know, the incidence of plasmids in Antarctic soil bacteria is not high (Ray *et al.* 1991) and no plasmids, involved in alkanes assimilation capability, have been detected in *Acinetobacter* (Singer & Finnerty 1984). This supports the assumption that in *Acinetobacter* strains genes encoding alkanes assimilation are located on the chromosome (Asperger & Kleber 1991).

In spite of the particular environmental conditions of Antarctica, *Acinetobacter* ADH-1 does not differ, in general features, from other members of the genus isolated so far from the Antarctic continent. Thus, we think that this strain could be useful in the design of "*in situ*" or "ex situ" bioremediation processes where temperature is one of the limiting factors. This does not mean that ADH-1 can be applied only in the Antarctic continent. On the contrary, since ADH-1 is a psychrotrophic (and not an obligately psychrophilic) strain, this microorganism could be useful in decontamination techniques in any cold or temperate climates, as the major part of the petroleoum producer area of our country (Patagonia and Tierra del Fuego) where oil spills and oil wastes represent a serious and unsolved environmental problem (Mendoza *et al.* 1995). Obviously, further research and other psychrotrophic bacterial strains with different degradative properties are needed in order to obtain, in the future, a microbial consortia capable of an efficient hydrocarbon degradation.

Finally, we would like to remark on the potential that is contained in the permanently cold areas of Antarctic environment, not only at basic microbiological research level but, as reviewed recently (Margesin & Schinner 1994, Feller

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et al. 1996) in the field of applied microbiology as well.

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