Potential for Biodegradation of Hydrocarbons by Microorganisms Isolated from Antarctic Soils

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Seventeen pure aerobic microbial isolates were obtained from soil samples of three regions of Antarctica: Casey Station, Dewart Island and Terra Nova Bay. Most of them were gram positive coryneform bacteria. Isolates were tested for their ability to grow on mineral salt agar plates supplemented with one of the following model *n*-alkanes or aromatic hydrocarbons: hexane, heptane, paraffin, benzene, toluene, naphthalene and kerosene. Cell hydrophobicity, the ability to produce anionic glycolipids and extracellular emulsifying activity were also determined and assessed on the basis of growth of soil isolates on hydrocarbons. This study revealed degraders with broader abilities to grow on both types of hydrocarbons, good production of glycolipids and emulsifying activity. On this basis, a mixed culture of strains is proposed, which may find application for bioremediation at temperate temperature of soil environments polluted with different hydrocarbons.

Key words: Hydrocarbons, Hydrophobicity, Emulsifying Activity

Introduction

The pristine environment of Antarctica has been impacted by human activity. Both alkanes and aromatic hydrocarbons are deposited on land as a result of field operations involving fuel storage and refueling of aircrafts and vehicles. Biodegradation is most often the primary mechanism for contaminant destruction including petroleum contaminants (Leahy and Colwell, 1990). Bioremediation, *i.e.* the enhancement of natural biological degradation processes, has been proposed for clean-up of oil-spills in Antarctic soils (Kerry, 1993) as cost-effective technology of removing contaminants. There have been a few studies on microbial oil degradation in Antarctic soils in which hydrocarbon-degrading microbes have been detected (Kerry, 1990; 1993; Aislabie *et al.*, 1998). It has been proposed that bacteria are the major colonizers of these oil-contaminated soils (Kerry, 1990). The activity of microbial types naturally present can be enhanced by bioremediation techniques which include increased aeration of the polluted area and nutrient additions (Christofi and Ivshina, 2002; Ivshina *et al.*, 1998).

Bioremediation is limited by the poor availability of hydrophobic pollutants. Contaminated soils contain a separate non-aqueous-phase liquid (NAPL), which may be present as droplets or films on soil surfaces. Biodegradation takes place more readily when the target contaminants are dissolved in an aqueous solution, but many hydrocarbons are insoluble in water and remain partitioned in the NAPL. Hydrocarbon-degrading microorganisms, often bacteria, have developed different adaptations for utilizing poorly soluble substrates. Most of them produce biosurfactants, amphiphilic molecules of diverse chemical nature and molecular size and with effective surface-active and biological properties (Desai and Banat, 1997; Rosenberg and Ron, 1999). They help to disperse the hydrocarbons, increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability, thereby stimulating the growth of bacteria and the rate of bioremediation (Ron and Rosenberg, 2002). Supplementing the source of microorganisms capable of degrading particular pollutants (bioaugmentation) and the enhancement of desorption of pollutants from particulates using surfactants can increase hydrocarbon degradation (Ivshina *et al.*, 1998). The surface properties of bacteria are important in determining the fate of implanted bacteria in the soil environment because of their influence on the vertical transport, distribution and survival.

For the application of bioremediation in Antarctica, indigenous microbes are required since the

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Antarctic Treaty prohibits the introduction of foreign organisms. Furthermore, hydrocarbon-degrading microbes indigenous to Antarctic soils are adapted for growth and survival under the local conditions existing in soils, which, when thawed in summer, are typically cold, dry, low in nutrients, and often alkaline (Aislabie *et al.*, 1998).

In the present work, a number of microbial strains were isolated from soils of three regions of Antarctica, impacted or not by human activity. Some characteristics of the isolates were determined, such as the morphology, the capability to grow on some model hydrocarbons, hydrophobicity, production of surfactants and extracellular emulsifying activity. The aim of this study was to detect the best performing hydrocarbon degraders with good emulsifying activity and producing glycolipids. Based on this screening, a model consortium should be proposed for treatment of soil environments polluted with different hydrocarbons.

Materials and Methods

Isolation of microorganisms

Different Antarctic soil samples (Casey Station, Dewart Island and Terra Nova Bay) were used for microbe isolation. One gram of soil from each site was homogenized and rinsed with 20 ml of physiological solution. Serial dilutions were made and 0.05 ml of them were spread onto plates with appropriate media as beef extract-pepton agar; Kuster agar; Czapec agar; mineral agar No 1; maize extract agar No 6 (Gauze *et al.*, 1983). Cultivation was carried out at 4 °C, 12 °C and 28 °C. The cultures growing on some media were isolated, maintained on meat-peptone broth with 1.5% agar (MPA) at 4 °C and transferred monthly.

Morphological and physiological characterization of isolates

Isolates were examined after growth on MPA for Gram reaction and cell morphology. Detail morphology of the strains on solid and liquid media was observed by light microcopy. Some biochemical characteristics of the cultures as growth on carbon sources, pigment production and temperature range and others were studied according to Bergey's Manual of Determinative Bacteriology.

Media and growth conditions

All cultivations were performed in mineral salts medium (MSM) which contains (g l^{-1}): $(NH_4)_2SO_4$ (2.0); KH_2PO_4 (6.0); $Na_3C_6H_5O_7 \cdot 2\ H_2O$ (1.0); $MgSO_4 \cdot 7\ H_2O$ (0.1), supplemented with 2 mM $CaCl_2$ (pH 7.0).

Isolated pure cultures were tested for their ability to grow on solid MSM with 1.5% agar and with 2% of one of the following model hydrocarbons: alkanes – hexane, heptane and paraffin; aromatics – benzene, toluene and naphthalene; and kerosene, a mixture of aromatic hydrocarbons and alkanes. Hydrocarbons were sterilized by filtration through 0.2-μm membrane filters (Millipore Corp., USA). Agar plates were incubated for 20 to 30 days at 20 °C or 28 °C.

Isolate A-13 was cultivated in 300 ml Erlenmeyer flasks containing 40 ml liquid MSM supplemented with 2% (v/v) of one of the following hydrocarbons: heptane, paraffin, benzene and kerosene as a sole source of carbon and energy. As inoculum was used culture after 24 h of cultivation in liquid meat-peptone broth (MPB). Flasks were incubated shaking (130 rpm) for 16 days at 25 °C. Growth was monitored by measuring the optical density at 570 nm (OD_{570}).

Detection of glycolipids

Soil isolates were screened for the ability to produce anionic glycolipids on solid media. Blue agar plates containing cetyltrimethylammonium bromide (CTAB) (0.2 mg ml^{-1}; Sigma) and methylene blue (5 μg ml^{-1}) in MSM were used to detect extracellular glycolipid production (Deziel *et al.*, 1996). Glycolipids were observed by the formation of dark blue halos around the colonies.

Cell surface hydrophobicity test

Cell hydrophobicity was measured by microbial adherence to heptane according to a method of Rosenberg *et al.* (1980) with slight modification. The cells were washed twice and resuspended in PUM buffer, pH 7.1 (22.2 g $K_2HPO_4 \cdot 3\ H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7\ H_2O$ and distilled water to 1000 ml), to an initial absorbance of the cell suspension at 550 nm of 0.5–0.6. The cell suspension (1.2 ml) with heptane (0.2 ml) was vortexed in a test tube at high speed for 2 min and equilibrated for 1 h. The optical density of the bottom aqueous phase was then measured at 550 nm. Hydrophobicity was expressed as the per-

centage of adherence to the hydrocarbon calculated as follows: 100 × (1 – OD of the aqueous phase/OD of the initial cell suspension).

Emulsifying activity

The emulsifying activity of the supernatant fluids after growth of soil isolates on glucose was determined using the test of Berg *et al.* (1990) with slight modification. Samples (0.2 ml, after suitable dilution) were mixed with 0.5 ml of TM buffer (20 mM Tris [tris(hydroxymethyl)-aminomethane]/HCl buffer, pH 7.0 and 10 mM $MgSO_4$) and then 0.1 ml of kerosene was added. The tubes were then vortexed at room temperature and at high speed for 1 min. The turbidity of the water phase was measured at 550 nm after standing for 1 min. One unit of emulsifying activity was defined as the amount of the emulsifier producing an absorbance of 1.0 at 550 nm in the assay (EU ml^{-1}).

Results and Discussion

Isolation of soil microorganisms

Seventeen morphologically different aerobic microbial colonies were isolated from Antarctic soil samples. On solid media some strains showed rod morphology with v-shaped structures. Dependent on the growth phase in liquid media (exponential or stationary) the cells passed from rods to cocci. Morphological and some biochemical properties of the strains give us a means to determine them as coryneform bacteria (Table I). Other strains have features of actinomycetes and belong to following genera: *Micromonospora*, *Nocardia* and related *Nocardioform* group.

Table I. Characterization of soil isolates.

Isolate	Isolation temperature	Gram staining	Strain identification
A-1	12 °C	Gram positive	*Coryneform*
A-2	12 °C	Gram positive	*Coryneform*
A-3	12 °C	Gram positive	*Coryneform*
A-4	28 °C	Gram negative	nonidentified
A-5	28 °C	Gram positive	*Coryneform*
A-6	28 °C	Gram positive	*Nocardia* sp.
A-7	12 °C	Gram positive	*Coryneform*
A-8	28 °C	Gram positive	*Nocardioform*
A-9	12 °C	Gram positive	*Coryneform*
A-10	28 °C	Gram positive	*Micromonospora* sp.
A-11	12 °C	Gram positive	*Coryneform*
A-12	12 °C	Gram negative	nonidentified
A-13	28 °C	Gram negative	nonidentified
A-14	4 °C	Gram positive	*Coryneform*
A-15	12 °C	Gram positive	*Nocardioform*
A-16	28 °C	Gram positive	*Coryneform*
A-17	28 °C	Gram positive	*Nocardioform*

Growth of isolates on solid media with some model hydrocarbons

Isolated pure cultures of soil microorganisms were screened for their ability to grow on MSM with 1.5% agar and with 2% of each one of the model *n*-alkanes or aromatic hydrocarbons (1% for naphthalene) used as a sole carbon source (Table II). As can be seen, only four isolates, A-5, 6, 7 and 12 were unable to grow on the hydrocarbons used. All other isolates showed growth (more or less) on both aromatic and aliphatic hydrocarbons tested, especially strains A-1, 3, 8, 13–15. Ten of the isolates formed dark blue halos on agar plates indicating a production of anionic glycolipids (Table II).

Biosurfactants are produced by a wide variety of microorganisms and have different natural roles in the growth of microorganisms (Ron and Rosenberg, 2001). Most of biosurfactants are different types of glycolipids (Desai and Banat, 1997). Secreted surface-active compounds improve cell growth and bioavailability of hydrophobic compounds thus accelerating their degradation (Ron and Rosenberg, 2002).

Hydrophobicity and emulsifying activity of soil isolates

Glucose-grown soil isolates were tested for their cell surface hydrophobicity to heptane and for extracellular emulsifying activity. Of the 17 strains studied, strain A-7 had the lowest (7.6%), and strain A-3 had the highest (85.6%) hydrophobicity values (Table III). Strains could divide into three groups according to their hydrophobicity to heptane: with high hydrophobicity (above 40%) – isolates A-3, 8, 10; with middle hydrophobicity (between 20 and 40%) – isolates A-4–6, 9, 11, 13, 15, 17 and with low hydrophobicity (under 20%) – isolates A-1, 2, 7, 12, 14, 16.

All isolates showed extracellular emulsifying activity after growth on glucose (Table III). It was highest for isolates A-3–5, 12, 13 (in the range 1.2–2.6) although strains A-5 and A-12 did not show growth on all the hydrocarbons used.

Hydrophobicity is an important factor in the initial adhesion of microorganisms to the interface between the NAPL and the aqueous phase. A

Table II. Growth and glycolipid production of microbial isolates from Antarctic soils on solid media with 2% of indicated hydrocarbons as a sole carbon source.

Isolate	Heptane	Hexane	Paraffin	Benzene	Toluene	Naphthalene	Kerosene	Glycolipid production
A-1	+	–	+	–	+	+	+ +	+
A-2	–	–	+	–	–	–	–	–
A-3	–	–	+	+	+ +	+ +	+ + +	+
A-4	+ +	–	–	–	+ +	+	+	–
A-5	–	–	–	–	–	–	–	–
A-6	–	–	–	–	–	+	–	–
A-7	–	–	–	–	±	–	–	–
A-8	+ +	+	+ + +	+	+ +	+	+	+
A-9	–	–	–	–	–	–	+	+
A-10	–	–	–	–	–	–	+	–
A-11	–	–	–	–	–	+	+	+
A-12	–	–	–	–	–	–	–	–
A-13	+ + +	+ + +	+ + +	+ + +	+ + +	+	+ + +	+
A-14	+	+	–	+	+	–	+ +	+
A-15	–	+	+	+	+	+	+	+
A-16	+	–	–	+	+	–	+	+
A-17	–	–	–	+	–	–	+	+

–: No formation of colonies observed.
+: Formation of weak colonies < 1 mm diameter.
+ +: Formation of colonies between 1 to 3 mm.
+ + +: Formation of colonies between 3 and 5 mm and more.
±: Means marginal growth.
Glycolipid production was registered as dark blue halos around the colonies.

Table III. Hydrophobicity and emulsifying activity of microbial isolates from Antarctic soils after growth on 2% glucose.

Isolate	Hydrophobicity (%)	*Emulsifying activity [EU ml^{-1}])
A-1	17.8 ± 2.1	0.59 ± 0.12
A-2	14.4 ± 4.5	0.54 ± 0.21
A-3	85.6 ± 5.5	2.38 ± 0.22
A-4	32.0 ± 2.6	1.20 ± 0.48
A-5	37.8 ± 2.5	1.22 ± 0.40
A-6	20.2 ± 1.5	0.60 ± 0.19
A-7	7.6 ± 1.1	0.90 ± 0.57
A-8	72.4 ± 2.5	0.32 ± 0.16
A-9	19.5 ± 3.2	0.77 ± 0.20
A-10	42.6 ± 3.4	0.48 ± 0.15
A-11	28.4 ± 2.8	0.69 ± 0.16
A-12	16.7 ± 7.2	2.57 ± 0.52
A-13	27.2 ± 1.8	1.42 ± 0.37
A-14	9.3 ± 1.6	0.56 ± 0,17
A-15	29.6 ± 3.6	0.32 ± 0.11
A-16	10.0 ± 3.9	0.31 ± 0.19
A-17	23.0 ± 4.1	0.60 ± 0.18

* Emulsifying activity to kerosene. EU: Emulsifying units as absorbance at 550 nm per ml.
Values are mean of three separate experiments ± s.d.

number of species of bacteria are able to degrade liquid hydrocarbons after adhering to the surfaces of droplets (Stelmack *et al.*, 1999). This direct contact between a bacterial cell and a target hydrocarbon can significantly increase the rate of diffusion into the cell, thereby enhancing growth and increasing the apparent rate of dissolution of the hydrocarbon. Such correlation between high initial cell hydrophobicity and good growth on hydrocarbons exists for A-3 and A-8 isolates, while isolates A-1, 4, 13–16 growing on the hydrocarbons tested were with middle or low hydrophobicity.

In the present study, all soil isolates with different initial hydrophobicity showed extracellular emulsifying activity. Isolates with highest activity, A-8 and A-13, were very different in their initial hydrophobicity. Similar observations have been reported by other authors that both hydrophilic and hydrophobic bacteria were able to produce surfactants when grown on glucose or hexadecane (Neu and Poralla, 1990; Bouchez-Naitali *et al.*, 1999).

Table IV. Hydrophobicity and emulsifying activity of strain A-13 after growth in liquid mineral salts media with 2% of each one of the hydrocarbons used as a sole carbon source.

Hydrocarbon	*Growth (OD_{570})	Hydrophobicity (%)	**Emulsifying activity [EU ml^{-1}]
Glucose (control)	0.58 ± 0.15	27.2 ± 2.4	1.42 ± 0.37
Heptane	0.05 ± 0.02	82.9 ± 2.8	0
Paraffin	0.56 ± 0.18	57.5 ± 3.2	0.74 ± 0.08
Kerosene	1.90 ± 0.10	43.9 ± 1.9	0.64 ± 0.10
Benzene	0.10 ± 0.04	67.6 ± 3.1	0

* Strain was cultivated at 25 °C with agitation 12 d; on kerosene – 16 d; on glucose – 2 d.
** Emulsifying activity to kerosene. EU: Emulsifying units as absorbance at 550 nm.
Mean values from three separate experiments are given ± s.d.

Cultivation of strain A-13 with some hydrocarbons in batch system

Strain A-13 with the best growth on agar plates with some model hydrocarbons was cultivated in liquid MSM with 2% of each one of the hydrocarbons used as a sole carbon source. The strain showed best growth on kerosene and extracellular emulsifying activity after growth on paraffin and kerosene. It was observed an increase in cell hydrophobicity after cultivation of the strain on each one of the hydrocarbons used (Table IV) which can be induced to change in the presence of combination of both excreted surfactants and slightly soluble substrate (Zhang and Miller, 1994).

During growth on soluble carbon source glucose high emulsifying activity of isolate A-13 was observed indicating production of biosurfactants. This suggests a broader role for biosurfactants than just hydrocarbon uptake. A likely possibility is the more general participation in adhesion and de-adhesion interactions between microorganisms and interfaces (Neu, 1996).

The results presented here will be particularly useful in choosing strains for environmental applications involving the implantation of microorganisms in the soil matrix (bioaugmentation). As contaminated sites usually contain heterogeneous hydrocarbons, it is promising to use for bioaugmented clean-up strains with broad abilities to grow on different hydrocarbons. For this purpose a model consortium including isolates A-1, 3, 8, 13–15 with good emulsifying activity and producing glycolipids was proposed for hydrocarbon waste treatment of polluted soil environments.

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