Diesel oil and PCB-degrading psychrotrophic bacteria isolated from Antarctic seawaters (Terra Nova Bay, Ross Sea)

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Fifty-seven Antarctic marine bacteria were examined for their ability to degrade commercial diesel oil as the sole organic substrate at both 4 °C and 20 °C. Based on the preliminary screening, two isolates (B11 and B15) with high capacity to degrade diesel oil were selected and their biodegradation efficiency was quantified by gas chromatographic analysis. As expected for psychrotrophs, diesel oil biodegradation was slower at 4 °C than at 20 °C. The two strains also mineralized the C_{28} n-paraffin octacosane at 20 °C and polychlorinated biphenyls (PCBs) at 4 °C and 20 °C.

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Thanks to their metabolic potential and wide biodiversity micro-organisms play a fundamental role in making biodegradation processes more efficient, and in transforming recalcitrant macromolecules into chemical substances more amenable to further metabolization. Psychrotrophic bacteria, which are adapted to a wide temperature range and to other fluctuating conditions such as low nutrient availability, low water activity and high pressure, may have important advantages in biotechnological applications (Gounot 1991; Mohn et al. 1997) such as the removal of pollutants from cold environments.

Cripps (1990) claimed that hydrocarbons, both aliphatic and aromatic, present in the Southern Ocean are above all of a biogenic nature and can be considered as part of the natural background. However, human activities in Antarctica (such as tourism, research and fishing, all of which require fossil fuels for transport and energy), potentially make petroleum hydrocarbons the most likely source of pollution in Antarctic ecosystems (Bícego et al. 1996; Cripps & Shears 1997; Ferguson et al. 2003). Several studies have shown that pollution is actually a localized phenomenon closely connected to human activity (Karl 1993; Tumeo & Wolk 1994).

Evaporation and photo-oxidation represent the first steps in the disappearance of hydrocarbons from seawater, while microbial degradation, along with weathering processes, is the ultimate fate of oil at sea (Leahy & Colwell 1990). In the past few years new species of marine bacteria capable of biodegrading hydrocarbons have been isolated from various marine areas (Delille & Vaillant 1990; Bícego et al. 1996; Delille et al. 1998; Gentile et al. 2003; Yakimov et al. 2003).

However, natural microbial degradation is slow and difficult in the marine environments due to the low concentrations of nitrogen, phosphorous salts and oxygen, and the formation of water-in-oil emulsion and tar balls. Furthermore, biodegradation is often limited by the antimicrobial effect of petroleum components (Leahy & Colwell 1990; Heipieper et al. 1992), the toxicity of the water-soluble oil fraction and—more indirectly—by a general inhibition of biological processes due to the increase of UV radiation with decreasing protection by the ozone layer.

Pollutants other than hydrocarbons have been detected in Antarctic seawater. Among these, polychlorinated biphenyls (PCBs) are long-term persistent compounds widely utilized in industrial applications for their heat resistance, lipophilicity

and relative inertness (Master & Mohn 1998; Vaillancourt et al. 2003).

In the present study, two psychrotrophic marine strains from Antarctica (namely, B11 and B15) were selected from 57 isolates because they had a strong ability to degrade diesel oil, as demonstrated by their noticeable growth in a culture medium containing this substrate as sole carbon source. Their biodegradative potential on diesel oil as well as on PCBs was estimated by gas chromatography, in order to assess whether they could be a useful tool for the removal of these organic pollutants from marine environments.

Materials and methods

Bacterial strains

The psychrotrophic bacterial strains used in this study were previously isolated (Maugeri et al. 1996) from Antarctic seawater samples (Terra Nova Bay, Ross Sea) collected along the water column (0 - 200 m) in two fixed stations: 23 from Mergellina (MER: 74° 41' 33" S - 164° 07' 15" E; about 250 m from the coast) and 34 from Santa Maria Novella (SMN: 74° 43' S - 164° 16' E; in the middle of Terra Nova Bay, about 10.5 km from MER). From among the total of 152 bacterial strains isolated by Maugeri et al. (1996), these 57 strains were selected, on the basis of their lipolytic activity on Tween 80 (Sierra 1957).

The isolates are maintained at 4 °C on slopes of marine agar (from Difco) and routinely streaked on agar plates from tubes every six months to control purity and viability. Antarctic strains are also preserved by freezing cell suspensions at –80 °C in marine broth (Difco) to which 20 % (vol/vol) glycerol is added.

Growth conditions

Hydrocarbon degradation was screened on a mineral liquid medium (Mills et al. 1978) supplemented with commercial diesel oil at a final concentration of 1 % (vol/vol). Ten-millilitre portions of the medium were placed in pre-sterilized screw cap tubes and 100 µl of pre-sterilized commercial diesel oil was added to each portion. The medium was inoculated with 100 µl of a bacterial suspension previously prepared in distilled water supplemented with 3 % (wt/vol) NaCl. The effect of temperature on the biodegradative activity was determined by cultivation at 4 °C and 20 °C for one month and one week, respectively, on a rotary shaker operated at 100 rpm in order to guarantee a continuous supply of oxygen. Turbidity was the positive indicator. Uninoculated control tubes were incubated in parallel to monitor abiotic losses of the substrate. All assays with uninoculated controls were performed in duplicate.

Two strains (B11 and B15) seemed to have a strong degradation effect on diesel oil at both 4 °C and 20 °C. Based on these preliminary results, their ability to mineralize both the long-chain alkane octacosane (C_{28}) and a PCB mixture (Aroclor 1242) was examined. The mineral liquid medium was supplemented with octacosane or PCBs at a final concentration of 0.2 % (wt/vol and vol/vol, respectively). Removal of these substrates was recorded after incubation at 4 °C and 20 °C. Both strain B11 and strain B15 had been isolated from samples taken at the Santa Maria Novella sampling station at a depth of 5 m.

Gas chromatographic analysis

Gas chromatographic analysis was performed for strains B11 and B15 grown on diesel oil, octacosane (C_{28}) and PCBs. In addition, strain D41 (isolated from the Santa Maria Novella station at the depth of 200 m) and strain D48 (isolated from surface water from Mergellina) were randomly chosen among those that made the culture medium turbid at 4 °C, but did not seem to grow on diesel oil as the sole carbon source at 20 °C.

Following the incubation periods, the cultures of each tube were extracted twice with 20 ml of *n*-hexane as a solvent by using separatory funnels to remove the cellular material. The extracts were transferred to tared vials. The volume of each extract was adjusted to 100 ml by adding more *n*-hexane, and vials were kept at 4 °C until the gas chromatographic analysis was carried out.

Biodegradation of hydrocarbons was quantified by quantitative gas chromatographic analysis using a DANI 8521-a GC equipped with an SE-54 fused silica capillary column (25 m × 0.32 mm i.d.; 0.45 µm film thickness) and a flame ionization detector. Hydrogen (1 kg/cm^2) was used as the carrier gas. The temperature program consisted of an initial oven temperature of 50 °C for 5 min increased at a rate of 10 °C/min to 280 °C for 10 min and then isothermal for 10 min. Injector and detector temperatures were maintained at 280 °C. The splitting ratio was 1:60.

PCB degradation was determined by quantitative gas chromatography using a Carlo Erba Mega 5300 GC equipped with an electron capture detector and an SPB-5 fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness). Helium (1.5 kg/cm^2) was used as the carrier gas. The initial oven temperature was 150 °C, increased to 230 °C at a rate of 2 °C/min and then increased to 280 °C at a rate of 10 °C/min (10 min hold). The temperature of the injector and the detector were 250 °C and 280 °C respectively. Nitrogen (1.5 kg/cm^2) was the gas at the detector. The splitting ratio was 1:25.

The degradation was expressed as the percentage of substrate degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples (external standard technique). The biodegradation efficiency (BE), based on the decrease in the substrate concentration as a whole, was evaluated by using the following expression:

$$BE\ (\%) = 100 - (A_s \times 100/A_{ac})$$

where A_s is the total area of peaks in each sample, A_{ac} is the total area of peaks in the appropriate abiotic control and BE (%) is the biodegradation efficiency.

Results

During this study, all but six of the 57 lipolytic isolates screened grew on diesel oil as the sole source of carbon and energy. Twenty-one of the strains from MER and 30 of the strains from SMN showed an ability to biodegrade diesel oil at at least one of the temperatures tested (Fig. 1). The number of diesel oil-degrading bacteria isolated from each sampling depth in relation to the temperature of incubation is shown in figure 2. Twenty-eight isolates (13 from MER and 15 from SMN) degraded diesel oil at 4 °C exclusively (assessed after a month of incubation), while six (all from SMN) did so only at 20 °C (assessed after a week of incubation). Diesel oil was observed to be utilized at both temperatures by 17 strains only: nine of them were isolated from SMN and eight from MER. Among these isolates strains B11 and B15 gave the highest turbidity. Quantitative gas chromatographic analysis allowed the estimation of their biodegradation

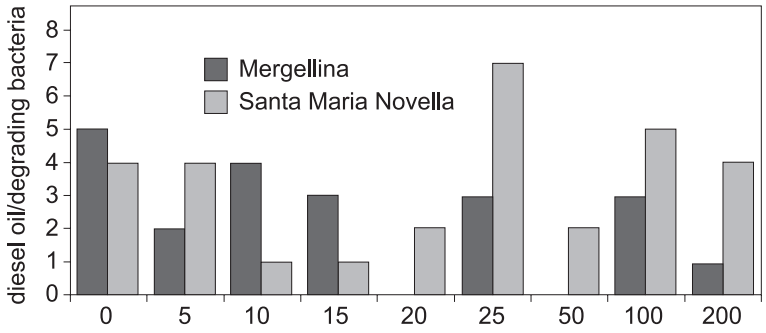

Fig. 1. Distribution of the 51 diesel oil-degrading bacteria in relation to their origin (sampling station and depth). Strains utilizing the substrate at at least one of the temperatures are included.

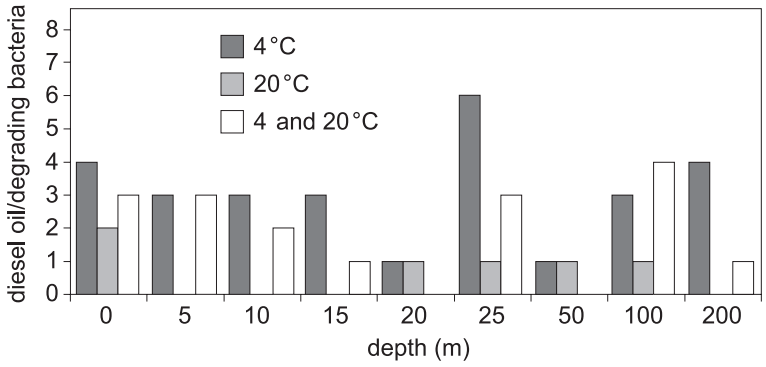

Fig. 2. Distribution of diesel oil-degrading bacteria in relation to the incubation temperature and to the depth. The appearance of turbidity was used as the positive indicator.

efficiency, which was expressed as the percentage of substrate degraded (Fig. 3). Very little difference was observed between the degradation of diesel oil by strain B11 at 4 °C and 20 °C. In culture medium inoculated with strain B15, 66.7 % of the diesel oil had disappeared after the one-month incubation at 4 °C and 50.5 % had disappeared after one week at 20 °C. A significant diesel oil mineralization was observed for strains D41 and D48 at 20 °C (66.10 % ± 2.55 [mean ± SD] and 56.15 % ± 0.49 [mean ± SD], respectively). The biodegradative activity was confirmed by the calculation of the C_{17}:pristane and C_{18}:phytane ratios (Table 1).

Strains B11 and B15 also mineralized the C_{28} *n*-paraffin octacosane at 20 °C and PCBs at 4 °C and 20 °C as the sole carbon source. Strain B11 utilized 31.9 % and 62.8 % of the PCB mixture at 4 °C and 20 °C, respectively, while 22.8 % and 30.8 % of the substrate disappeared from the culture medium inoculated with strain B15 after incubation at 4 °C and 20 °C, respectively.

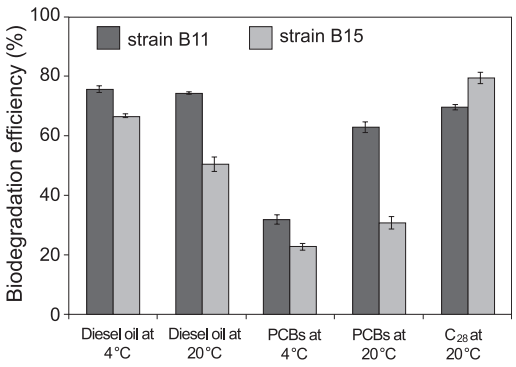

Fig. 3. Percentage of diesel oil, PCB and C_{28} biodegraded by the strains B11 and B15.

Table 1. C_{17}:pristane and C_{18}:phytane ratios.

Sample	Incubation temperature (°C)	C_{17}:pristane ratio	C_{18}:phytane ratio
Abiotic control	4 and 20	2.2	2.1
Strain B11	4	1.7	2.2
Strain B15	4	1.2	1.5
Strain B11	20	1.3	1.5
Strain B15	20	1.1	1.1
Strain D41	20	1.4	1.6
Strain D48	20	0.7	0.5

Discussion

Diesel oil is a complex combination of hydrocarbons deriving from the distillation of crude oil and represents an excellent substrate in the study of hydrocarbon biodegradation thanks to its composition (Bicca et al. 1999). Diesel oil contains 2000 to 4000 different hydrocarbons (Marchal et al. 2003) with a carbon number ranging approximately from C_9 to C_{20}, including paraffin, olefins, naphtha and aromatic compounds.

During the screening for the degradation of diesel oil, only 6 of the 57 lypolitic isolates did *not* mineralize the substrate, despite their ability to hydrolyse Tween 80. This highlights the strong relationship between lipolytic activity and the biodegradation of diesel oil as previously shown by Mills et al. (1978).

The amounts of diesel oil mineralized by strains B11 and B15 at both the temperatures tested were very similar. Since extraction for the gas chromatographic analysis was carried out after a month of culture at 4 °C and after a week at 20 °C, this finding suggests that both strains degraded diesel oil more slowly at 4 °C than at 20 °C, as expected for psychrotrophs. This is in line with the effect of temperature on the rate of hydrocarbon metabolism by micro-organisms: metabolism decreases with decreasing temperature.

The significant diesel oil mineralization by strains D41 and D48 at 20 °C should be highlighted. These strains had been randomly chosen amongst those that showed growth at 4 °C but not at 20 °C as indicated by the turbidity of the culture medium. In this kind of qualitative screening, the assumption of hydrocarbon utilization cannot be based only on the appearance of turbidity; instead a chromatographic analysis of the culture is needed. On the other hand, Mills et al. (1978) showed that in liquid media it is possible that micro-organisms only grow in the oily phase, producing neither visible growth in the oil–water interface nor visible turbidity in the water phase. Mills and co-workers suggested that in such cases the quantity of proteins in the culture should be determined because production of protein in quantities greater than 30 µg/l is correlated to easily visible turbidity. However, as is well known, one must take into account that the rate of hydrocarbon degradation is not always directly related to the bacterial growth.

The C_{17}:pristane and C_{18}:phytane ratios were generally characterized by lower values than the

abiotic control. Pristane, widely employed as an internal standard for the analysis of hydrocarbon samples, is characterized by a high degree of persistence (Watkinson & Morgan 1990). Thus, its disappearance from the culture medium demonstrates the occurrence of biodegradation.

The biodegradation efficiencies on PCBs were lower than those observed for diesel oil. This is not surprising as PCBs are highly recalcitrant to biodegradation. Lower incubation temperature severely limited PCB degradation. In fact, the removal of PCB by bacterial degradation was higher at 20 °C. After only a week of incubation at 20 °C the disappearance of PCBs was two-fold higher than at 4 °C after a month.

Yakimov et al. (1999), searching for bioemulsifier-producing bacteria, selected the strains B11 and B15 and identified them at molecular level as marine variants of *Rhodococcus fascians*. Psychrotrophic alkane-degrading members of the genus *Rhodococcus* sp. have also been isolated from a number of cold environments (Whyte et al. 1996, 1998, 2002; Bej et al. 2000). Rhodococci have been found to utilize a variety of xenobiotic compounds as carbon source (Seto et al. 1995; Larkin et al. 1998) and to possess a variety of alkane-catabolic pathways (Whyte et al. 1998; Smits et al. 1999; Andreoni et al. 2000).

The results obtained confirm that culturable Antarctic bacteria are capable of degrading hydrocarbons, both at 4 °C and at 20 °C. As many authors state (Atlas 1981; Bertrand et al. 1993; Whyte et al. 1997; Delille & Delille 2000), the exposure of the microbial community to hydrocarbons is responsible for its potential to oxidize these compounds and, consequently, for the number of micro-organisms able to utilize them. Also Delille & Vaillant (1990), who did in situ and laboratory experiments to investigate the effect of crude oil on the growth of sub-Antarctic marine bacteria, found that the addition of hydrocarbons always induced a marked, rapid increase in the bacterial population. It is therefore surprising that hydrocarbon-degrading marine bacteria were isolated from both superficial and deep seawaters with hydrocarbon pollution levels as low as those of the Antarctic. Thus, the occurrence of these bacteria in Terra Nova Bay could probably be related to the presence of hydrocarbons of natural origin rather than to the impact deriving from human activities in Antarctica.

In conclusion, the ability of psychrotrophic marine bacteria of Antarctic origin to utilize diesel oil and PCBs as the sole carbon source was demonstrated. Although the experiments were performed in batch culture under stable conditions different from those of natural environments, this kind of screening is an essential step in the evaluation of bacteria biodegradation efficiency. In accordance with Whyte et al. (1998), our results suggest that psychrotrophic microorganisms, which clearly utilize a wide variety of hydrocarbons at temperatures ranging from 0 °C to 30 °C, may be better suited for in situ bioremediation in both temperate and cold environments than mesophiles or psychrophiles.

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