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Original article

Molecular and physiological characterisation of psychrotrophic hydrocarbon-degrading bacteria isolated from Terra Nova Bay (Antarctica)

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Abstract

A set of 21 Antarctic marine bacteria isolated from the Ross Sea and able to utilise diesel fuel as the sole carbon and energy source was characterised. Isolates were analysed by amplified 16S rDNA restriction analysis using the enzyme *Alu*I, resulting in two different groups corresponding to different bacterial species. These species were assigned to the genera *Rhodococcus* and *Alcaligenes*, on the basis of 16S rDNA sequencing. This low degree of inter-specific biodiversity was parallel to a low intra-specific biodiversity, as shown by Random Amplified Polymorphic DNA analysis. Then, a 550-bp DNA fragment coding for the inner region of alkane mono-oxygenase was PCR-amplified from the genome of each strain. The phylogenetic analysis of the sequence of the putative AlkB protein coded for by the amplified DNA fragment revealed that these *alkB* genes were very likely inherited by horizontal gene transfer. Lastly, the analysis of the biodegradation ability of four strains revealed two different strategies of hydrocarbon uptake, mediated either by bio-surfactants and peculiar of *Rhodococcus* isolates, or by membrane modifications and shown by *Alcaligenes* isolates. In order to understand the interrelationships between hydrocarbon-degrading isolates, the dynamics of two strains, belonging to *Rhodococcus* and *Alcaligenes*, grown together in a co-culture was also followed over a seventeen days period.

Keywords: *n*-alkanes; 16S rDNA; RAPD; ARDRA; *alk* genes; Biosurfactants

1. Introduction

Aromatic and aliphatic hydrocarbons of oil origin are serious environmental pollutants due to their persistence and high toxicity in biological systems including the marine habitats. Once released into marine environment hydrocarbons are partially degraded by endemic microbial communities, the most toxic and refractory fraction settle into sediments, resulting in damages to marine ecosystems.

Many bacteria thrive on aromatic and/or aliphatic hydrocarbons as the sole carbon and energy source. These bacteria are isolated not only from contaminated sites but also from pristine ones. Little is known about

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the genetics of the *n*-alkane biodegradation. Indeed, only the alkane degradative genes from *Pseudomonas* have been described in detail. The *alk* system identified and characterised in the *Pseudomonas putida* strain Gpo1 represents the most studied alkane hydroxylase model ([23] and references therein). In this bacterium, the first step of *n*-alkane oxidation is catalysed by a three-component alkane hydroxylase complex, consisting of a non-hem integral-membrane alkane monooxygenase and two soluble proteins, a rubredoxin and a rubredoxin reductase. A similar three-component alkane hydroxilase complex, which consists of an alkane mono-oxygenase (AlkM), a rubredoxin (RubA), and a rubredoxin reductase (RubB) has been recently characterised in *Acinetobacter* sp. strain ADP1 [8,16,17]. Much less is known about the alkane degradative system in other bacteria, such as gram positive ones. However, an *n*-alkane hydroxylase system from two *Rhodococcus* strains referred to as NRRL B-16531 and Q15 and isolated from different geographical sites, has been more recently characterised [27]. Both *Rhodococcus* strains possess at least four alkane monooxygenase gene homologs (*alkB1*, *alkB2*, *alkB3* and *alkB4*). In both strains the *alkB1* and *alkB2* homologs belong to an *alk* gene cluster, which also includes genes coding for two rubredoxins (*rubA1* and *rubA2*; *rubA3* and *rubA4*), a putative TetR transcriptional regulatory protein (*alkU1*; *alkU2*), and, in the *alkB1* cluster, a rubredoxin reductase (*rubB*). The *alkB3* and *alkB4* homologs were found as separate genes that were not part of alkane gene clusters. The presence of the alkane gene clusters in the two *Rhodococcus* strains is reminiscent of other multiple-degrative-enzyme systems reported in *Rhodococcus*. Bacteria belonging to the genus *Rhodococcus* are increasingly recognised as very good candidates for the biodegradation of hydrocarbons, because of their: (1) ability to degrade a wide range of organic compounds [2,12]; (2) hydrophobic cell surfaces; (3) biosurfactant production [29]; and (4) ubiquity and robustness in the environment [11,25].

A crucial step in hydrocarbon degradation is represented by the uptake of these molecules by bacterial cells; in fact, the efficient degradation requires the solubilization of hydrocarbon molecules. For this purpose, different bacteria developed different strategies to interact with hydrophobic molecules [5].

The presence of pollutants has been demonstrated also in Antarctic seas; it is mainly due to the atmospheric and oceanic circulation, but anthropogenic hydrocarbon contamination deriving from tourism, research and fishing activities is increasing more and more [3,4]. Concurrently, recent studies have demonstrated that micro-organisms able to degrade hydrocarbons are present also in Antarctic seas. These bacteria show physiological and biochemical adaptations that allow them to be metabolically active in extreme conditions, thanks to their production of cold adapted enzymes and polyunsaturated fatty acids ([9] and references therein). The study of these bacteria is of particular interest because low temperatures (ranging from −1.8 to 2 °C), characteristic of Antarctica, increase the diesel fuel viscosity reducing volatility of short chain alkane (<C10). Besides, many medium- or long-chain compounds (>C10) become solid; for this reason pollutants persist in the environment for a long time. However, nothing is known about the interrelationships existing between microorganisms belonging to the same Antarctic hydrocarbon-degrading microbial community.

The aim of this work was to perform a molecular characterisation of diesel fuel-degrading bacteria isolated from the Ross Sea surface waters (Terra Nova Bay, Antarctica), using a strategy based on a combination of different techniques [6], which has been previously and successfully applied to the study of microbial communities from different environments [7,13]. Furthermore, we try to understand the relationships among hydrocarbon-degrading bacteria coming from the same Antarctic microbial community.

2. Materials and methods

2.1. Bacterial strains and media used

Superficial seawater samples were collected during the XV Italian Expedition in the 1999/2000 Antarctic summer from the inlet Road Bay (Terra Nova Bay, Ross Sea). In order to determine the viable bacterial counts and the frequency of hydrocarbon-degrading bacteria, tenfold serial dilutions of the water samples were plated onto both solidified Plate Count Agar (PCA: 5 g tryptone, 2.5 g yeast extract, 1 g glucose, 24 g NaCl, 16 g agar per litre of distilled water) and minimal medium MMV (24 g NaCl, 1 g $MgSO_4$ $7H_2O$, 0.7 g KCl, 2 g KH_2PO_4, 3 g Na_2HPO_4, 1 g NH_4NO_3, 16 g agar per litre of distilled water) [14], containing 0.4% (v/v) of diesel fuel (Esso Italiana) as the sole carbon and energy source (MMG). Diesel fuel was previously filtered trough a 0.22 μm-pore-size filter (Sartorius) for sterilization and particle removal.

After incubation at 4 °C for 18 days, the colonies from the MMG plates were picked at random and restreaked at least three times on the same medium until pure cultures had been obtained.