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Isolation of Surfactant-Resistant Bacteria from the Surface Microlayer

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Abstract—Bioremediation efforts sometimes rely on surfactants to enhance hydrocarbon bioavailability. However, synthetic surfactants are toxic to some degrading microorganisms. In this study we screened a natural surfactant-rich compartment, the sea surface microlayer (SML), for surfactant-resistant bacteria, using enrichment cultures of sodium dodecyl sulfate (SDS) and cetyl trimethylammonium bromide (CTAB). A random set of isolates obtained from enrichment cultures was tested by PCR for the *Pseudomonas* genus marker *gacA* gene and the *ndo* gene, encoding for PAH-degrading enzyme naphthalene dioxygenase. Results indicate a high abundance of surfactant resistant bacteria in the SML. An exponential increase in the number of culturable bacteria (7–8 logs) was observed during the course of the enrichment culture. *gacA*-targeted PCR revealed that 30% of the surfactant-resistant isolates corresponded to Pseudomonads. However, amplification of sequences corresponding to *ndo*-gene involved in PAH-degrading pathways was not yet successful.

Keywords: sea surface microlayer, bacterioneuston, surfactant, bioremediation, *Pseudomonas*

INTRODUCTION

Petroleum is a major source of energy, and of products for industry and daily life. The transport of petroleum across the world is frequent, and consequently, the potential for oil spills is significant. Bioremediation is considered an efficient, cost effective and versatile alternative to physical and chemical treatment of hydrocarbon contamination (Sun *et al*., 2008) and bacteria are particularly suitable for biodegradation because they adapt to a the wide variety of carbon sources and electron acceptors (Vasileva-Tonkova and Galabova, 2003). However, because of the hydrophobic nature of hydrocarbons, the microbial degradation is limited by their reduced solubility and consequent low bioavailability (Plante *et al*., 2008).

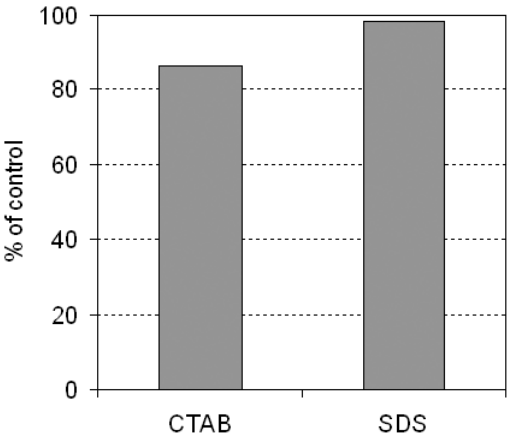

Fig. 1. Mean proportion (%) of surfactant resistant bacteria in selective media (PY medium + 8 mM of SDS or 1 mM of CTAB) in relation to the counts in non-selective (PY medium) medium, in the initial water sample.

A possible process of enhancing the availability of oil hydrocarbons is the application of surfactants. Surfactants reduce surface and interfacial tension and lead to the formation of microemulsions in which hydrocarbons can solubilise in the water, increasing their bioavailability (Plante *et al.*, 2008). Because of their amphipathic nature, surfactants are known to alter the structure and function of the cellular membrane, induce cellular lysis (Glover *et al.*, 1999) and alter the structure and functions of important bacterial enzymes (Dong *et al.*, 1997; Gonçalves *et al.*, 2003). Broadly speaking, cationic surfactants are considered to be the most toxic, while anionic are less toxic and more active against Gram-positive than Gram-negative bacteria. Non-ionic surfactants are often considered non-toxic. Some studies have shown that surfactant addition often fails to enhance the biodegradation of hydrophobic compounds because the toxicity of the surfactants decreases the viability and activity of bacterial cells (Sun *et al.*, 2008). Therefore, a pre-requisite for effective surfactant-enhanced biodegradation is that the degradative microorganisms are not adversely affected by the surfactant (Plante *et al.*, 2008).

The surface microlayer (SML) is the uppermost millimetre of the water column and bacteria living in this compartment (bacterioneuston) face a challenging environment in terms of exposure to solar irradiation, accumulation of pollutants and surface tension (Agogué *et al.*, 2005). However, bacterioneuston is often reported to reach higher cell abundance than the underlying bacterioplankton, suggesting an effective adaptation to this extreme environment (Agogué *et al.*, 2005; Franklin *et al.*, 2005). Being biosurfactants the most common mechanism by which microorganisms deal with interfacial challenges, the SML may represent a particular environment where surfactant-resistant

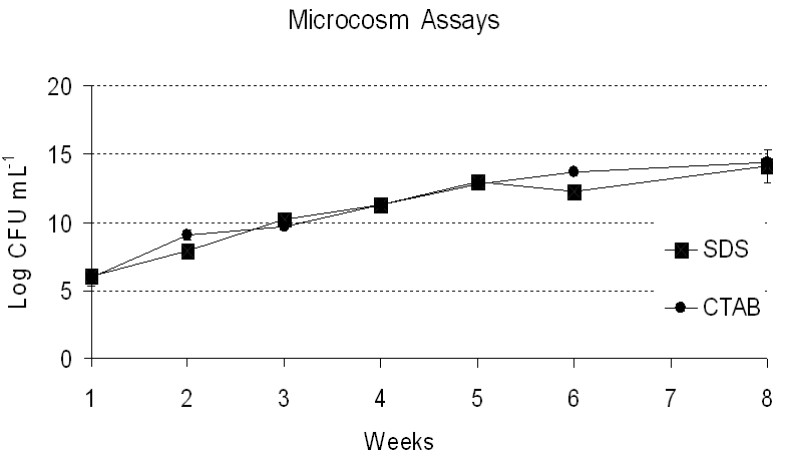

Fig. 2. Variation of the mean CFU counts in enrichment cultures of bacterioneuston in microcosm amended with 1 mM CTAB or 8 mM SDS.

bacteria are expected to be positively selected. The objective of this work was to obtain and characterize bacterioneuston isolates with enhanced resistance to surfactants in order to further explore their degradative capacities in a perspective of bioremediation.

MATERIALS AND METHODS

SML samples were collected at locations next to a shipping harbour in the Ria de Aveiro, a shallow (mean depth 1 m) coastal lagoon ecosystem located in northeast Portugal, using the glass-plate method as described by Agogué *et al*. (2004). The glass plate was dipped in the water and left to drip for approximately five seconds. Immediately after, the layer of SML was collected by passing the glass plate through a grid.

For the preparation of enrichment cultures, samples of SML water were spread on triplicate plates of PY (peptone-0.1% and yeast extract-0.01%, diluted in brackish water salinity 17 gL^{-1}) containing 70 mM SDS (Sodium dodecyl sulphate, Bio-Rad), 1 mM CTAB (cetyl trimethylammonium bromide, Sigma) or without surfactant. These samples were considered to represent the initial inoculum used in the enrichment cultures. In parallel, two microcosms were prepared using 200 mL of SML sample and 800 mL of basal saline medium (BSM) (adapted from Amirmozafari *et al*., 2007). The anionic surfactant SDS or the cationic surfactant CTAB were added to each of the microcosms in approximate critical micelle concentrations (CMC) of 8 mM and 1 mM, respectively. The cultures were incubated at room temperature for two weeks on a rotary shaker operating at 90 rpm. Aliquots were collected weekly and spread on PY plates amended with the approximate CMC concentrations of SDS or CTAB (3 replicates

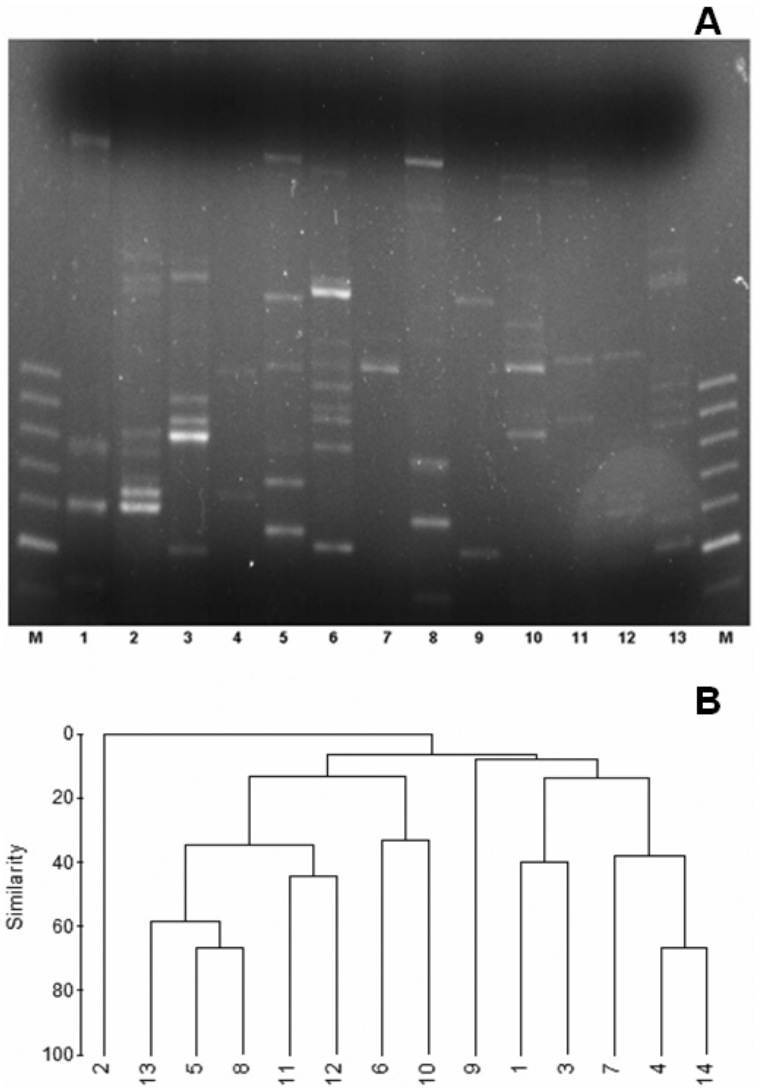

Fig. 3. Results of molecular typing of a set of 13 randomly selected isolates conducted by repetitive sequence PCR using the BOX A1R primer; A) Electrophoresis gel of PCR-BOX products. B) Dendrogram of the similarity index of Bray-Curtis of the PCR-BOX profiles.

of each). After 3 days of incubation at 25°C, bacterial abundance was estimated by colony counting.

Selected colonies were purified by successive streaking on surfactant amended PY plates (minimal 3 streaks). DNA was extracted as described by Henriques *et*

al. (2004) from pure-cultures. Molecular typing of a set of 13 randomly selected isolates was conducted by repetitive sequence PCR using the BOX A1R primer (BOX-PCR fingerprinting) (Rademaker *et al*., 1998). Similarity matrices of densitometric curves of the gel tracks were calculated using the Pearson Product Moment Correlation Coefficient followed by tree construction using UPGMA algorithm. The presence of *gacA* (*Pseudomonas* genetic marker) and *ndo* genes (naphthalene dioxygenase genes) was assessed by PCR. The primers used were NAPH-1F and NAPH-1R (Gomes *et al*., 2007) for *ndo* and GACA-1F and GACA-2 (De Souza *et al*., 2003) for *gacA*. The presence of PCR products of 306 (*gacA*) (Cébron *et al*., 2008) and 896 base pairs (*ndo*) (Gomes *et al*., 2007) was assessed by electrophoresis in ethidium bromide-stained 1.5% agarose gels at 80V for 30 min and visual inspection under a UV transiluminator.

RESULTS AND DISCUSSION

A high relative abundance of culturable surfactant-resistant bacteria was found in estuarine bacterioneuston (Fig. 1). CFU counts in SDS and CTAB amended media at T_0, were 98% and 87% of the counts obtained in non-selective medium, respectively but this difference was not statistically significant (ANOVA > 0.05). In contrast to what is reported in the literature (Van Hamme *et al*., 2006), CTAB does not seem to be more toxic to SML bacteria than the anionic surfactant SDS. The high abundance of surfactant resistant bacteria detected could possibly be explained by the adaptation of bacterioneuston to the high levels of naturally and anthropogenically derived surfactants occurring at the SML due to its interfacial location and amphiphilic nature (Kozarac *et al*., 2005; Salter *et al*., 2009). Such observation also identifies the SML as a source of surfactant-resistant bacteria.

The abundance of culturable bacteria in the enrichment cultures, assessed by colony counts in PY medium (Fig. 2), showed an exponential increase (7–8 logs) during the duration of the experiment, in the presence of both types of surfactants as a result of the selective enrichment of bacteria capable of withstanding the surfactant toxic effects and probably utilizing it as a carbon source for their growth.

The BOX-PCR analysis of the set of isolates obtained from the enrichment cultures is presented in Fig. 3. The similarity between the isolates was lower than 70% and therefore they were considered as different strains. This observation suggests a high diversity within the surfactant-resistant bacterioneuston community.

The results of the amplification of *gacA* gene fragments indicated that 30% of the surfactant resistant isolates belonged to *Pseudomonas* genus. Pseudomonads are ubiquitous and well known for their versatility in the biodegradation of a wide range of organic compounds (Jovčić *et al*., 2009), including surfactants (Jovčić *et al*., 2009) and PAHs (Bamforth and Singleton, 2005). PCR targeting *ndo* genes was negative for the same set of isolates. However, the potential for PAH degradation should not be excluded because the protocol for the detection of hydrocarbon-degrading genes can be considerably improved.

CONCLUSION

A diverse set of surfactant resistant bacteria was retrieved from the SML of the estuarine system Ria de Aveiro. PCR analyses of isolates indicated that *Pseudomonas* is well represented in the culturable fraction of surfactant-resistant bacterioneuston, but did not confirm PAH-degrading genotypes, indicated by the inability to amplify naphthalene dioxygenase genes. Further phenotypic and genotypic identification with different primers may unveil hydrocarbon degradation capacities which may become very useful in the bioremediation efforts of hydrocarbon contaminated sites, especially in the Ria de Aveiro.

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