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Research article

Response of the bacterial community in oil-contaminated marine water to the addition of chemical and biological dispersants

Camila Rattes de Almeida Couto [a], Diogo de Azevedo Jurelevicius [a], Vanessa Marques Alvarez [a], Jan Dirk van Elsas [b], Lucy Seldin [a, *]

[a] Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
[b] Microbial Ecology Department, University of Groningen, Groningen, The Netherlands

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ABSTRACT

The use of dispersants in different stages of the oil production chain and for the remediation of water and soil is a well established practice. However, the choice for a chemical or biological dispersant is still a controversial subject. Chemical surfactants that persist long in the environment may pose problems of toxicity themselves; therefore, biosurfactants are considered to constitute an environmentally friendly and effective alternative. Nevertheless, the putative effects of such agents on the microbiomes of oil-contaminated and uncontaminated marine environments have not been sufficiently evaluated. Here, we studied the effects of the surfactant Ultrasperse II® and the surfactin (biosurfactant) produced by *Bacillus* sp. H2O-1 on the bacterial communities of marine water. Specifically, we used quantitative PCR and genetic fingerprint analyses to study the abundance and structure of the bacterial communities in marine water collected from two regions with contrasting climatic conditions. The addition of either chemical surfactant or biosurfactant influenced the structure and abundance of total and oil-degrading bacterial communities of oil-contaminated and uncontaminated marine waters. Remarkably, the bacterial communities responded similarly to the addition of oil and/or either the surfactant or the biosurfactant in both set of microcosms. After 30 days of incubation, the addition of surfactin enhanced the oil-degrading bacteria more than the chemical surfactant. However, no increase of hydrocarbon biodegradation values was observed, irrespective of the dispersant used. These data contribute to an increased understanding of the impact of novel dispersants on marine bacteriomes before commercial release into the environment.

1. Introduction

The use of dispersants - amphipathic molecules that are able to reduce water/oil surface/interfacial tensions - is regarded as a promising tool to promote oil dispersion and remediation of water and soil. The tool may also be useful in the main stages of the oil production chain, such as extraction, transportation, and storage (Smyth et al., 2010). There are two general classes of dispersants: (i) chemically synthesized molecules (surfactants) and (ii) biological molecules that are produced as metabolic byproducts from microbial growth (biosurfactants) (Nitsche and Pastore, 2002; Smyth et al., 2010).

Surfactants that persist long in the environment may lead to the accumulation of toxic or harmful substances, causing serious environmental problems (Abd-Allah, 1995; Deschenes et al., 1996). The accumulation of surfactants in river sediments (Rico-Rico et al., 2009), in marine water and sediments (Petrovic et al., 2002) and in infiltrated ground water (Field et al., 1992) has already been demonstrated. Many of these surfactants were found in sufficient concentrations to constitute toxicity problems to aquatic organisms (Ankley and Burkhard, 1992; Uzoigwe and Okpokwasili, 2012). Moreover, surfactants have also been found to be deleterious to various bacteria (via e.g., membrane lysis, DNA damage and starvation) (Ivankovic et al., 2009; Lima et al., 2011). Therefore, the use of surfactants is usually regulated by environmental safety agencies. In Brazil, only two chemical surfactants (Corexit EC9500 and Ultrasperse II®) are licensed by The National Council of Environment (CONAMA; Resolution 269) for treating oil-contaminated

* Corresponding author. Laboratório de Genética Microbiana, Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco I, Ilha do Fundão, CEP 21941.590, Rio de Janeiro, RJ, Brazil.
E-mail address: lseldin@micro.ufrj.br (L. Seldin).

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marine environments.

The use of Corexit EC9500 in marine waters has extensively been documented after the Deep Water Horizon Oil (DWH) spill (Zhao et al., 2015). This surfactant, a water-soluble mixture containing hydrocarbons, glycols and dioctyl sulfosuccinate (DOSS) (Baelum et al., 2012), has been considered to be toxic to different marine organisms (Gardiner et al., 2013). Furthermore, Kleindienst et al. (2015) demonstrated that the viability of some of the main marine oil-degrading bacterial groups (*Marinobacter* and *Acinetobacter* genera) was reduced, even with low concentrations of Corexit EC9500. Oppositely, to our knowledge, there are only few studies on the effect of Ultrasperse II® - a mixture of alcohol, alcohol sulphate and the fatty ester ethoxylate - on the marine biome. The use of Ultrasperse II® is restricted to some countries and/or used by oil companies, often with intellectual property protection. However, toxicity of Ultrasperse II® to marine fish has been demonstrated (Cruz et al., 2012). Such surfactant toxicity has incited worldwide attempts to reduce the 'after effects' of these molecules.

On the other hand, biosurfactants are often considered as environmentally friendly and effective alternatives to petroleum dispersants (Blyth et al., 2015). They offer a number of advantages over chemical surfactants, such as biodegradability due to their simple chemical structure, environmental compatibility, low toxicity and activity under conditions of extreme temperatures, pH and salinity (Kapadia and Yagnik, 2013; Silva et al., 2014). Among the well-known biosurfactants, surfactin, a lipopeptide that is mainly produced by *Bacillus subtilis*, is considered one of the most powerful and effective biosurfactants described so far (Barros et al., 2007). Moreover, surfactin, like other biosurfactants, has antimicrobial properties which make it very useful as a biocide (Ginkel, 1989; Korenblum et al., 2008; Couto et al., 2015).

Previous studies have demonstrated that biosurfactants are less toxic than synthetic surfactants to some invertebrate species (Edwards et al., 2003). However, the environmental risks posed by biosurfactants, assessed through their effect on microbial communities of oil-contaminated and uncontaminated marine environments, have not been sufficiently evaluated (Franzetti et al., 2006; Silva et al., 2014).

Here, we compare the effect of a chemical surfactant, Ultrasperse II® with that of a biosurfactant, i.e. the surfactin produced by *Bacillus* sp. H2O-1 (Korenblum et al., 2005), on the bacterial communities of oil-contaminated and uncontaminated marine environments. We hypothesized that the use of biosurfactant may result in a lower impact on bacterial communities, including the oil-degrading bacterial community. To test this hypothesis, we constructed microcosms using water samples contaminated with crude oil and with the addition of either a surfactant or a biosurfactant. These water samples were collected from Grumari beach (Rio de Janeiro, Brazil) and from Schiermonnikoog beach, Island of Schiermonnikoog (Groningen, The Netherlands), two regions with contrasting climatic conditions (tropical and temperate weather, respectively). Quantitative PCR and genetic fingerprint analyses were used to study the abundance and the structure of the aforementioned bacterial communities, allowing a better understanding of their response when exposed to the Ultrasperse II® and to the surfactin produced by *Bacillus* sp. H2O-1.

2. Materials and methods

2.1. Dispersant sources

The surfactin from *Bacillus* sp. H2O-1, a strain originally isolated from an oil reservoir in Brazil and previously described by Korenblum et al. (2005), was produced and purified as previously described by Nitschke and Pastore (2006). Ultrasperse II® was obtained from Oxiteno, São Paulo, Brazil.

2.2. Sample sites and construction of the microcosms

Water samples (5 L) from saline ecosystems (3.5% of salt) were collected from Grumari beach in Rio de Janeiro, Brazil (23°2′59″S 43°31′35″W), and from Schiermonnikoog Island, located in The Netherlands (53°29′14″N 6°14′3″E). The water temperature from Grumari beach and Schiermonnikoog Island was 22 °C and 18 °C, respectively. The microcosms were constructed in triplicate using 25 ml of water from Grumari beach (GB) or Schiermonnikoog Island (SI). These microcosms were submitted to different treatments as follows: a) control - microcosms containing only water - W; b) microcosms with water and the addition of biosurfactant - WB (surfactin, 40 μg/ml); c) microcosms with water and the addition of chemical surfactant - WS (Ultrasperse II®, 1 μl/ml); d) microcosms with water contaminated with crude oil - WO (1% v/v); e) microcosms with water contaminated with crude oil and the addition of biosurfactant - WOB; and f) microcosms with water contaminated with crude oil and the addition of chemical surfactant - WOS. The amount of dispersant used was based on a previous experiment where the emulsification index (EI) was calculated for each dispersant. The EI% was calculated based on the ratio of the height of emulsion layer and the total height of the liquid [EI% = (emulsion/total h) x 100]. We here used the highest dilution of the dispersant that was still able to give an EI equal to or higher than 30%. The oil samples were supplied by Petrobras in Brazil and by Royal Dutch Shell in The Netherlands. Both were considered medium oils. The microcosms were incubated at 20 °C, under shaking conditions (75 rpm) and in the dark, and temporal analyses were performed at 0 (immediately after the construction of the microcosms using only the original water samples) and after 5, 15 and 30 days of incubation (considered as t5, t15 and t30, respectively).

2.3. DNA extraction

The content of each microcosm was filtered through a Millipore membrane (0.45 μm), and the total DNA was extracted using FastDNA® Spin Kit for Soil (BIO 101 Systems, Ohio, USA) and then stored at 4 °C prior to PCR amplification.

2.4. PCR amplification of bacterial 16S rRNA encoding gene

The reaction was performed using the pair of primers F968 and 1401R-2b (Brons and Van Elsas, 2008) in a 25 μl-mixture containing about 10 ng of DNA, 100 nM of each primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 1.25 U *Taq* DNA polymerase (Stoffel, Applera, Connecticut, USA), 5 μl of 5X PCR buffer supplied by the manufacturer. The amplification conditions were as follows: initial denaturation of double-stranded DNA for 5 min at 94 °C; 10 (touchdown) cycles consisting of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C with a decrease in the annealing temperature of 0.5 °C per cycle; 25 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C; and an extension for 30 min at 72 °C. The products were analyzed by electrophoresis in 1.4% agarose gels, followed by ethidium bromide staining (1.2 mg/L ethidium bromide in 1X TAE buffer - 20 mM Tris-acetate, pH 7.4, 10 mM acetate, 0.5 mM disodium EDTA).

2.5. Denaturing gradient gel electrophoresis (DGGE) and statistical analyses

DGGE analysis was carried out as described previously (Muyzer et al., 1993) using the Ingeny PhorU2 apparatus (Ingeny International BV, The Netherlands). PCR products were loaded onto 8% (w/v) polyacrylamide gels in 1X TAE buffer. Polyacrylamide gels