

Chemotactic responses to gas oil of *Halomonas* spp. strains isolated from saline environments in Argentina

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ABSTRACT

In this study, two halophilic bacterial strains isolated from saline habitats in Argentina grew in the presence of gas oil. They were identified as *Halomonas* spp. and *Nesterenkonia* sp. by 16S ribosomal RNA sequencing. Chemotaxis towards gas oil was observed in *Halomonas* spp. by using swimming assays.

Keywords: Halophiles, *Halomonas*, hydrocarbon degradation, chemotaxis

RESUMEN

Respuesta quimiotáctica hacia gas oil de cepas de *Halomonas* spp. aisladas de ambientes salinos de Argentina. En el presente trabajo se aislaron dos cepas bacterianas halófilicas a partir de muestras obtenidas en ambientes salinos de Argentina, que crecieron en presencia de gasoil como única fuente de carbono. Las cepas aisladas se identificaron como *Halomonas* spp. y *Nesterenkonia* sp. mediante secuenciación del gen del ARN ribosomal 16S. En ensayos de *swimming*, las cepas del género *Halomonas* spp. mostraron una respuesta quimiotáctica hacia el gas oil.

Palabras clave: Halófilos, *Halomonas*, degradación de hidrocarburos, quimiotaxis

Bioremediation takes advantage of the ability of microorganisms to detoxify natural environments by degrading and using organic contaminants such as carbon and energy sources. Similarly to what happens in other ecosystems, saline/hypersaline environments are affected by contamination as a result of industrial effluents with high salt concentrations that are commonly produced by several industries like the leather and hydrocarbon (HC) industries. Since halophilic microorganisms are physiologically adapted to grow at high salt concentrations, they are expected to be more efficient for contaminant removal/degradation than non-halophiles. Thus, halophiles represent a valuable resource to remediate contaminated saline environments and effluents.

During the last decade, biodegradation of pollutants in saline environments was demonstrated and various halophilic and halotolerant microbes responsible for such activity were identified. These include: cyanobacteria (1), the halotolerant bacteria *Halomonas* spp. which degrades chloroaromatic compounds and phenols (3, 7), the haloarchaeon *Halobacterium* sp. isolated from a HC contaminated site (7) and members of genera *Haloarcula* and *Haloferax* that have been isolated from different types of hypersaline environments including salt lakes and crystallizer ponds from solar salterns (9, 14). Recently, Tapilatu *et al.* (12) reported the degradation of alkane and polyaromatic HC by HC-degrading haloarchaeal strains related to genera *Haloferax* and *Haloarcula* isolated from

polluted or uncontaminated environments. Several physicochemical and biological factors can affect the efficiency of bioremediation processes in natural environments and chemotaxis is one of such factors. Chemotaxis allows the cells to detect and respond to concentration gradients of different compounds. A number of bacterial strains have been described to be chemotactic towards the pollutants they are able to degrade (6, 10, 11), thus, the presence of chemotaxis is considered to be a desirable characteristic in bacteria involved in the biodegradation processes of contaminated environments (11). However, the chemotactic behaviour to HC has been poorly investigated in halophiles.

In this study, two halophilic bacterial strains were isolated and identified from saline environments in Argentina and they were tested for their ability to grow on gas oil (GO), a petroleum-derived fuel, and to respond chemotactically to this HC mix.

Two different saline habitats were examined for the occurrence of HC-metabolizing microorganisms: a salt brine located in Bahía Blanca estuary without HC contamination (38° 44' South 62° 16' West) and HC-contaminated sea water sampled from the harbor of Mar del Plata city (38° 00' South 57° 33' West). The samples obtained from the salt brine were initially grown in a saline medium containing (g/l): NaCl 100, MgSO₄ 7H₂O 14, KCl 1.4, sodium citrate 2.2, CaCl₂ 2H₂O 0.14; 0.05 % yeast extract (YE) (w/v) and 0.2% GO (v/v), at 37 °C with agitation. GO is

a complex mixture of HC which contains 75 % (v/v) alkanes (mainly hexadecane) and 25 % (v/v) aromatic HC (including naphthalenes and alkyl benzenes). This culture was used to inoculate (inoculation volume was 5 to 10% of the total culture) fresh medium containing only 0.025% YE (no sodium citrate) with and without GO (0.2 %) and incubated at 37 °C with agitation. Seawater samples were filtered through a 0.2 µm nitrocellulose membrane and the microorganisms retained on its surface were used to inoculate sterile seawater in the presence and absence of 0.2% GO and incubated at 25 °C for several days with agitation. Cultures grown with 0.5 % YE as the carbon and nitrogen sources were included as controls (data not shown). Cell growth was determined by measuring the optical density of the cultures at 600 nm (OD_{600}). Figure 1 A and B shows that cell growth was higher in the cultures containing GO compared to controls without GO (doubling times based on OD_{600} values were ~ 25 h in both samples). Although the cell density of the cultures was rather low, particularly in that inoculated with the HC-contaminated seawater, the results were reproducible in different experiments. Even though the concentration of GO along the growth curve was not directly measured, comparison of the time courses of cell growth in the presence and absence of GO suggests that the microbes in the saline samples were able to use the HCs present in GO. Gas chromatography analysis of the culture media before and after cell growth as well as the determination of cell growth in the presence of the individual HCs will help to identify the potential substrate/s metabolized by these halophiles. When observed under the microscope, very motile rod-shaped cells were visualized in the GO-cultures from the saline samples (salt brine and sea water) and this feature facilitated the develop-

ment of the chemotaxis assays. Unlike what happened in GO-containing cultures, almost no cells were detected in those without HC. To isolate the HC-degrading microorganisms, after several subcultures in the presence of GO both cultures were diluted and plated on the corresponding medium with GO and incubated until bacterial colonies were evident. Two different colonies were visualized in the salt brine sample, suggesting that at least two distinct microorganisms had been isolated. Liquid cultures from these isolates retained the ability to grow on GO (not shown) although only one of these isolates showed good motility. On the other hand, a single type of colony was isolated from the Mar del Plata harbor on GO-containing plates. To taxonomically identify the isolated halophiles, a fragment (1.3 kb) of the 16S ribosomal RNA (rRNA) gene was PCR-amplified and sequenced. With this aim, primers F43 Eco (5'-CGGAATTCAGGCCTAACACATGCAAGTC-3') and R1387 Eco (5'-CGGAATTCGGGCGGWGTGTACAAGGC-3') based on conserved sequences of bacterial 16S rRNA gene were used (8). Briefly, single colonies were suspended in 50µl sterile water and boiled for 5 minutes. These cell suspensions (5-10 µl) were used as template for the PCR reactions which contained: 0.25 µmoles/l of each primer (F43Eco and R1387Eco), 0.5 mmoles/l dNTPs, 0.5U *Taq* DNA polymerase (P-BL, Quilmes, Argentina), 1X *Taq* DNA polymerase buffer and 3 mmoles/l $MgCl_2$. Amplifications were carried out using the following temperatures: (94 °C 10 min) x 1; (95 °C 1 min, 55 °C 1 min, 72 °C 90 s) x 30, (72 °C 5 min) x 1. PCR products were fractionated on 0.8 % (w/v) agarose gels containing 0.5 µg/ml ethidium bromide and the DNA fragments of the expected size (1.3 kb) were purified and sequenced (Macrogen, Korea).

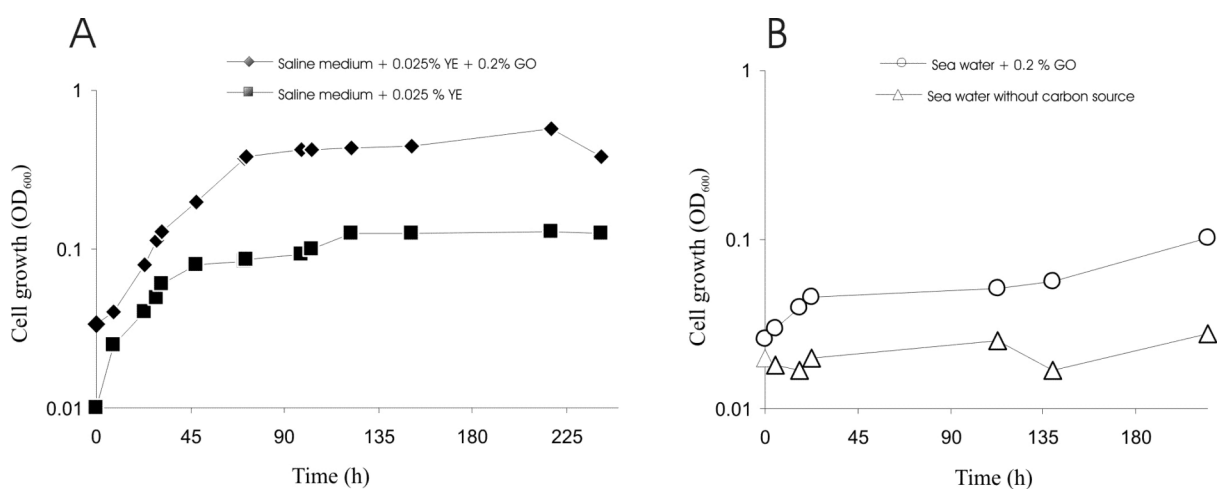


Figure 1. Growth curve of halophiles isolated from saline environments in the presence of GO. Samples taken from the saline environments were inoculated with agitation in the indicated media and temperature. (A) Salt brine sample. (B) Sea water sample. The results are representative of at least three independent experiments.

The nucleotide sequences of the amplified fragments were analyzed in Public Databases (<http://rdp.cme.msu.edu> and NCBI/BLAST). This allowed the identification of genus *Halomonas* (98 % identity to *Halomonas desiderata*), accession number HM454286 and *Nesterenkonia* (97 % identity to *Nesterenkonia aethiopica* and *Nesterenkonia halobia*) accession number HM454287 from the salt brine and genus *Halomonas* from the HC-contaminated sea water (97 % identity to *Halomonas* sp. ice-oil-302), accession number HM454288. *Halomonas* sp. belongs to the class gammaproteobacteria, family *Halomonadacea*, while *Nesterenkonia* sp. is included in the class *Actinobacteria*, order *Actinomycetales*, family *Micrococcaceae*.

The chemotactic behaviour of the halophilic bacterial strains isolated in this study was evaluated on swimming plates (13). In this assay, the attractant is added into the medium and bacteria generate and follow a gradient as they degrade and grow on the attractant. As a result a “ring of cells” is observed after incubation of the swimming plates for several hours at the indicated temperature. As shown in Figure 2 A and D, swimming rings were observed in both *Halomonas* spp. strains in the presence of GO indicating that they were chemotactic to the HC mix. This means that the *Halomonas* strains isolated in this study may have the ability not only to degrade but also to detect and actively swim towards some GO component/s ; otherwise, cells only growing on GO but not responding to it would accumulate in the site of inoculation, as observed for the *Nesterenkonia* sp. strain, which did not exhibit motility (data not shown). As expected, no responses

were evidenced in the absence of any carbon source (Figure 2, C and F). Although chemotaxis to aromatic HC has been reported in some non-halophilic bacteria (4, 10, 11), a chemotactic behaviour of microbes to alkanes (GO and hexadecane) has only been demonstrated for the bacterium *Flavimonas oryzihabitans* isolated from a HC-contaminated site from Argentina (6). The *Halomonas* spp. strains identified in this study also showed a chemotactic response towards YE (Figure 2 B and E), casamino acids, and citrate (data not shown). To the best of our knowledge, these findings represent the first report on chemotactic behaviour in the genus *Halomonas*. Although the ability of soil bacteria to degrade different toxic compounds is well documented, there is comparatively limited information on the catabolic activity of halophiles on organic pollutants and their potential to remediate contaminated saline ecosystems. Microbial diversity studies of several saline environments from Argentina have been carried out in the last years (2, 5). As an example, the study of community shifts in an exploited oil field with naturally high soil salinity near Comodoro Rivadavia in Patagonia (Argentina) identified a number of halophilic genera including *Halomonas*, *Dietzia* and *Alcanivorax* (5). Our study describes the isolation and identification of two halophilic bacteria related to genus *Halomonas* spp. and *Nesterenkonia* sp. from two different saline ecosystems of Argentina which grew on a mixture of HC (GO) and displayed chemotaxis to these compounds (*Halomonas* sp.). The occurrence of *Halomonas* spp. in different saline habitats of Argentina suggests that this genus may predominate in saline Argentine ecosystems

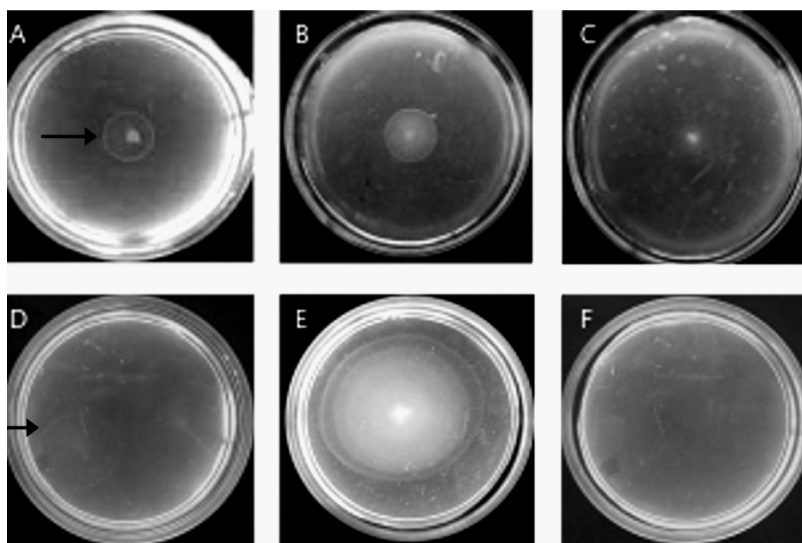


Figure 2. Determination of the chemotactic response towards GO of *Halomonas* spp isolated from saline environments in Argentina. Swimming plates of *Halomonas* spp isolated from salt brine (A, B, C) and/or sea water (D, E, F). Overnight cultures (4 μ l) were inoculated in the centre of soft agar plates containing GO (A, D), 0.05 % YE (B, E) or in the absence of a carbon source (C, F). Plates were photographed after 24 h of incubation at 30 °C. Arrowheads show swimming rings. The results are representative of at least three independent experiments.

or that these bacteria are easily isolated due to their ability to grow under laboratory conditions in the presence of toxic compounds. These findings suggest the potential of genus *Halomonas* as a biological tool for the remediation of HC-contaminated saline ecosystems.

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