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Tolerance of an Estuarine Halophilic Archaebacterium to Crude Oil and Constituent Hydrocarbons

T. M. Raghavan, I. Furtado

Department of Microbiology, Goa University, Goa 403 206, India

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Turnover of organic matter in estuaries is mainly controlled by heterotrophic activity of the diverse microbial members of resident communities (Scheiwer et al.1991). Salinity being an inherent characteristic of this econiche, the halophilic archaebacteria represent one of the dominant microflora (Boaden and Seed 1985) and hence possibly play a major role in the geochemical cycles of the estuaries. Extensive urbanisation and shipping activities expose any estuarine network to constant anthropogenic fluxes. Hence, globally, estuarine ecosystems are now envisaged to act as sinks for hydrocarbons and other pollutants.

Goa, on the West Coast of India, with several estuaries and tributaries running into the hinterland (Fig.1), lies in close proximity to the international oil tanker routes. The coastal waters of Goa showed presence of hydrocarbons way back in 1980 (Fondekar et al.1980). During the last decade, due to the expanding tourism and mining industries, there has been a 10-20-fold increase in the surface water traffic of barges, trawlers, passenger ferries, water boats, etc (the main contributors of petroleum hydrocarbons), besides the installation of several fuel stations in the areas flanking the Mandovi-Zuari estuarine network (Govt of Goa 1994).

Impact studies of hydrocarbon pollution in estuarine environments have been restricted to investigating the response of estuarine macro-organisms like filter feeding benthic invertebrates (Lee and Page 1997) and to single eubacterial isolates like *Pseudomonas aeruginosa* (Al-Hadhrami et al. 1997) or eubacterial consortia (Burns et al. 1997; Oudot and Dutrieux 1989). Reports on the response of estuarine halophilic archaebacteria to hydrocarbon pollutants are scarce (Bertrand et al. 1990; Kulichevskaya et al. 1991).

The present study reports the response of *Halobacterium* strain R_1 (MTCC3265 Microbial Type Culture Collection, India, hereafter referred as *Halobacterium* strain R_1), a halophilic archaebacterium, isolated from the Mandovi estuary (Fig.1) in Goa, India (Sequeira 1992) to crude oil and its constituent hydrocarbons.

Correspondence to: I. Furtado

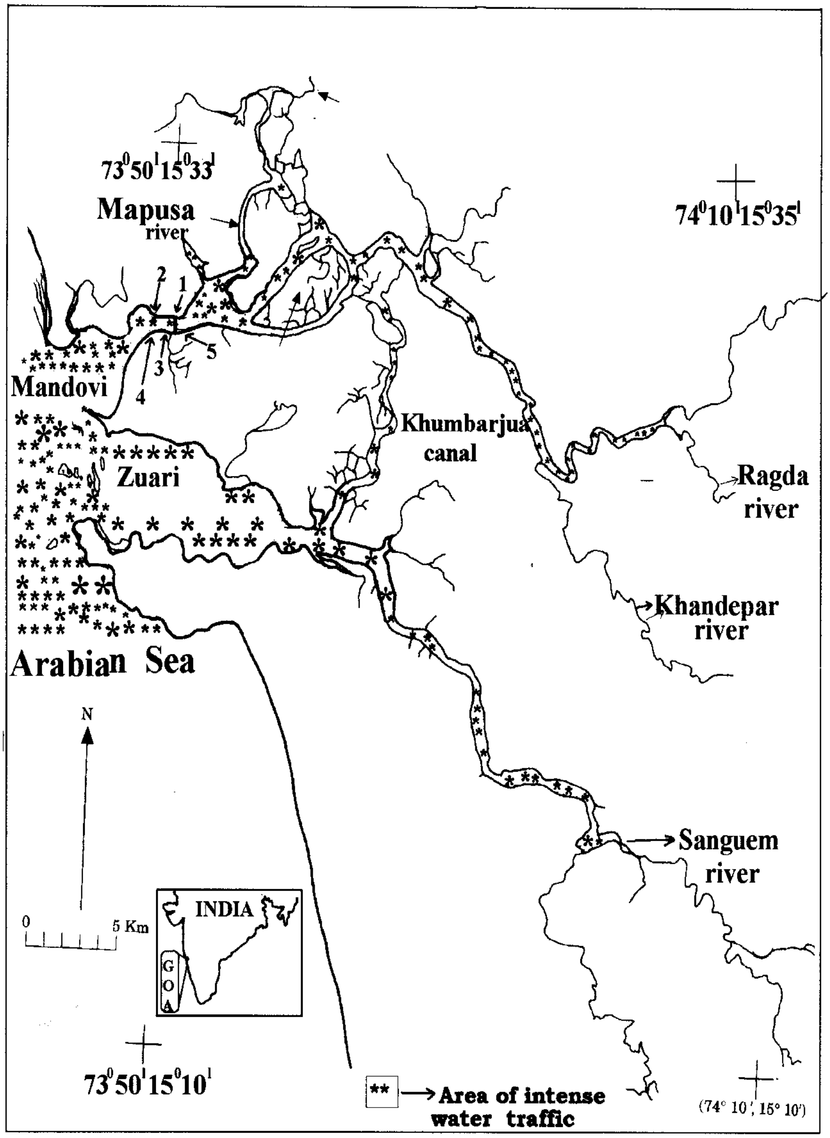

Figure 1. Mandovi-Zuari estuarine network

MATERIALS AND METHODS

Halobacterium strain R_1 was cultured on tryptone yeast extract medium (Steensland and Larsen 1968) containing 25% crude salt (NTYE), or on mineral salts medium (referred to as NSM) (Aguiar and Furtado 1996) and consisting of (g L^{-1}) of NaCl 200.0; $MgCl_2$. $6H_2O$ 13.0; $CaCl_2.6H_2O$ 1.0; KCl 4.0; $NaHCO_3$ 0.2; NH_4Cl 2.0; $FeCl_3.6H_2O$ 0.005; KH_2PO_4 0.5; distilled water 1000mL. The pH was adjusted to 7 using 1*M* KOH, 0.2% sterile glucose solution was added

(NGSM) and the cultures were then incubated at RT (28^0C-30^0C), on an orbital shaker (Scigenics, India) at 150 rpm. Culture was routinely maintained on NTYE or NGSM solidified with 20 gL^{-1} of agar (Hi-media, India).

Hydrocarbons used in the study were crude oil, petrol, diesel, kerosene, hexane (aliphatic), cyclohexane (alicyclic), benzene, toluene and xylene (aromatic hydrocarbons, abbreviated as BTX compounds). Tolerance to these hydrocarbons was determined by inoculating *Halobacterium* strain R_1 into flasks, each containing 50mL of NTYE or NGSM and 0.01% of a single hydrocarbon. Flasks were incubated at RT at 150 rpm and monitored periodically for absorbance at 600nm (UV-240 Visible Shimadzu Spectrophotometer, Japan). *Halobacterium* strain R_1 inoculated into appropriate media without hydrocarbon served as controls.

RESULTS AND DISCUSSION

As estuarine environments undergo transient phases of nutrient high and limitation (Ridd et al. 1997), we attempted to study the growth of response to *Halobacterium* strain R_1 in nutrient rich NTYE and nutrient limited synthetic NGSM.

Halobacterium strain R_1 grew in nutrient rich NTYE and synthetic NGSM, and showed a maximum absorbance of 2.2 and 1.6, respectively, at 600nm. In both media, the culture grew at salt concentrations between 5%-30% (Aguiar and Furtado1996). The requirement of a minimum of 5% salt concentration for growth corroborates the true halophilic nature of the estuarine isolate (Kushner 1985).

The culture attained an orange red and mauve coloration in NTYE and NGSM in 3 and 4 days, respectively. Pigmentation is an important characteristic of obligate halobacteria (Gibbons 1974). Acetone extracts of cells grown in NTYE and NGSM were identical with peaks at 450nm, 470nm, 495nm and 529nm, which are characteristic of carotenoids and bacterioruberins (Kushwaha et al. 1974).

Since *Halobacterium* strain R_1 was isolated from the Mandovi estuary (Fig.1), that has been receiving and continues to receive increasing inputs of crude oil and constituent hydrocarbons due to maritime activities (Govt. of Goa 1994; Mascarenhas 1999), we found it pertinent to look into the response of *Halobacterium* strain R_1 to crude oil and other toxic hydrocarbons both under nutrient rich as well as limited conditions.

As seen in Fig.2., the presence of any one of the hydrocarbons, except benzene/ hexane/cyclohexane decreased the growth of the culture in NTYE. A maximum of 44% decline in growth in the presence of petrol was followed by a decline of 40% in the presence of crude oil/diesel/kerosene. Of the BTX compounds, xylene and toluene decreased growth by 67% and 35% respectively, while, benzene had no effect.

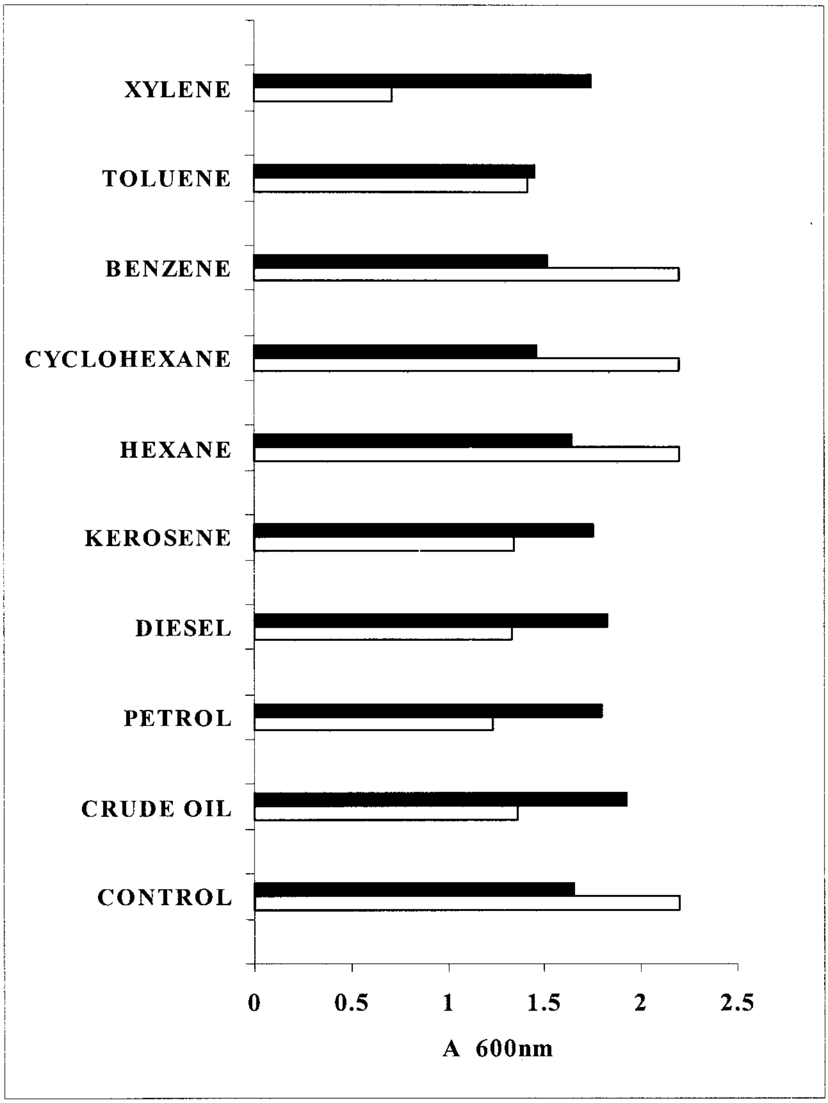

Figure 2. Growth of *Halobacterium* strain R_1 ■ NGSM; □ NTYE

In synthetic media (NGSM), on the other hand, the presence of crude oil/ petrol/ diesel/kerosene/xylene enhanced growth by 12%-16%. A maximum increase of 16% was seen with crude oil. There was no effect with hexane, but in the presence of toluene/ benzene/cyclohexane, there was a decline of 8%-14% in the growth of the culture. The growth of the culture in either of these media is unaffected by the presence of hexane.

Our above results clearly indicate that, the growth response of *Halobacterium* strain R_1 to the same hydrocarbon is governed by the environment, namely, nutrient excess/limitation. The hydrocarbons can be broadly categorized into three groups, based on the overall effects they have shown on the growth of *Halobacterium* strain R_1 (Fig.2):

a) benzene, cyclohexane and hexane: effecting very little change in either medium
b) toluene: reducing growth in both media
c) xylene, kerosene, diesel, petrol and crude oil : causing significant to very strong inhibition in rich medium, but slight improvement in standard medium

The United States Environmental Protection Agency's (USEPA 1987) quality criteria for water states that petroleum concentrations as low as 10 μgL^{-1} are toxic and detrimental to lobsters, aquatic larvae and zooplanktons. Furthermore, fuel oil at 9.4 μgL^{-1} is reported to be toxic to estuarine grass shrimp (Rayburn et al.1996).

In an earlier study, Al-Hadhrami et al. (1997), reported the *in vitro* tolerance of Pseudomonas *aeruginosa* to 0.1% of purified n-alkane components of crude oil. The survival and performance of such non-halophilic/halotolerant species in the halophilic environment of an estuary may however, be governed/influenced by salt gradients and hence restricted.

Our studies on the exposure of *Halobacterium* strain R_1, a representative of the dominant halophilic microflora of estuarine regions (Khandavilli et al.1999), to crude oil and other hydrocarbons is of significance, because, firstly, very few studies have been focussed on knowing the response of such estuarine halophiles to petroleum hydrocarbon pollutants. Secondly, as yet there are no reports that elucidate the response of microorganisms to hydrocarbon toxicants in nutrient rich and nutrient limited conditions, that would partly mimic the nutrient changes faced by resident microorganisms of estuarine econiches that are exposed to gradients of nutrient concentrations. It is noteworthy that *Halobacterium* strain R_1 tolerates 0.01% crude oil and its fuel derivatives, a concentration, which is 10 times higher than the toxicity level stated by the USEPA.

Benzene, toluene and xylene, the BTX components of crude oil, are known for their high toxicity (Dean 1985). These compounds being more water soluble than other hydrocarbons (Dunn et al.1986) are expected to be imbibed and bioaccumulated by filter feeding benthic invertebrates like mussels, oysters and bivalves (Lee and Page 1997) and thus in turn affect the food chain. The tolerance of *Halobacterium* strain R_1 to xylene in synthetic medium and to benzene in both the media, at a concentration of 0.01% each, is of interest, since the concentration is hundred fold higher than that reported by Myers et al. (1998) for wild marine bottomfish.

It is not clear, whether the observed 12%-16% increase in absorbance at 600nm, in case of cultures grown in the presence of crude oil/ petrol/ diesel/ kerosene/ xylene in NGSM, is truly due to an increase in the number of cells, or, is possibly, a mere enhancement of the pigmentation of the cells reflected as an increase in the observed absorbance. This increase in the growth of

Halobacterium strain R_1, therefore, needs to be investigated, particularly, in the light of the very strong inhibition effected by the same hydrocarbons in nutrient rich NTYE. Cooney and Shiaris (1984) have documented the utilisation and co-oxidation of aromatic hydrocarbons by estuarine microorganisms. Our results also suggest that *Halobacterium* strain R_1 may possibly be able to co-metabolize the hydrocarbons with glucose as the main substrate, or even utilise hydrocarbons as the sole sources of carbon, and, in the process, detoxify the same.

Halobacterium strain R_1 has also shown tolerance to heavy metals (Khandavilli et al.1999). It is possible that the observed multi-tolerance of *Halobacterium* strain R_1 to crude oil and constituent hydrocarbons (such as hexane, benzene, toluene), was acquired by the culture during adaptation to hydrocarbon pollution stress in the Mandovi estuary in Goa, India (Fig.1).

Furthermore, since *Halobacterium* strain R_1 belongs to the kingdom Archaea that has characteristic C_{20}, C_{40} isoprenoid lipids, it is possible that the utilisation of hydrocarbons is an inherent characteristic of such archaeal halophiles.

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