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Bacterial Chemotaxis to Effluent from a Rum Distillery in Tropical Near-Shore Coastal Waters

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Pseudomonas aeruginosa and *Vibrio cholerae* showed a strong positive chemotactic response towards rum distillery wastewaters (mostos) and a high oxygen uptake rate in the presence of this complex substrate. Rum slops stimulated only motility in *Aeromonas hydrophila* and *Escherichia coli*. The *A. hydrophila* and *E. coli* isolates were unable to oxidize mostos significantly.

The disposal of crude industrial effluents in tropical near-shore coastal waters is a rapidly increasing problem. One industry of tropical areas which has increased dramatically in the past 20 years is rum distillation (19). Biamon and Hazen (2) have recently reported that drastic changes take place in water temperature, dissolved oxygen, pH, content of inorganic and organic nutrients, and chlorophyll *a* concentration when rum slops are pumped into marine bays. The bay that they studied was Ensenada de Boca Vieja, near Catano, P.R., adjacent to San Juan harbor. This bay receives 1.4×10^6 liters of untreated effluent per day from the largest rum distillery in the world (4).

Rum distillery effluent can be characterized as hot, viscous, reddish brown, and odorous. It normally generates an anoxic and acidic environment at the plume outfall. At present, we do not know its exact chemical composition. However, we know that its inorganic fraction contains several minerals including heavy metals and high concentrations of nitrogen and phosphorus salts. The organic fraction is a complex mixture of simple sugars, polysaccharides, free amino acids, proteins, organic acids, and aromatic compounds (4).

Toxic and bacteriostatic properties of the undiluted effluent have been reported by others (7, 21–23). However, Biamon and Hazen (2) have found that densities of several potential pathogens, including *Aeromonas hydrophila, Escherichia coli, Vibrio cholerae*, and *Pseudomonas aeruginosa*, are high in the effluent plume ($>10^4$ CFU ml^{-1}). Indeed, the highest densities of these microorganisms have been measured at the sampling sites closest to the effluent outfall. Background counts (>200 m upcurrent) were always less than 10 CFU ml^{-1}. Moreover, survival studies with diffusion chambers have shown that some of these bacterial isolates can survive and multiply in the effluent (2; A. J. Lopez-Torres, M.S. thesis, University of Puerto Rico, Rio Piedras, 1982; N. Perez-Rosas and T. C. Hazen, unpublished data; L. J. Prieto and T. C. Hazen, unpublished data). Thus, rum distillery effluent can be an important source of potentially pathogenic bacteria in near-shore tropical environments.

In this study, we examined the behavioral response of the aforementioned bacteria toward a rum distillery effluent which is pumped into Ensenada de Boca Vieja from the largest rum distillery in the world. Chemotaxis and oxygen uptake were studied to explain the high densities of these organisms at the effluent outfall. The following alternatives were explored: (i) chemical components in the effluent that induce an oriented migration of specific microorganisms living in the surrounding waters toward the plume outfall and (ii) oxidation of nutrients present in the rum distillery wastewaters providing energy for bacterial growth and motility.

The four strains used in this study, *A. hydrophila, E. coli, V. cholerae*, and *P. aeruginosa*, were isolated from rum distillery wastewaters collected near the effluent plume at Ensenada de Boca Vieja, Catano, P.R. Bacterial suspensions for chemotaxis and oxygen uptake studies were prepared from 24-h cultures grown on Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). After incubation at 34°C for 18 to 20 h under continuous agitation, the cultures were centrifuged at 12,000 × *g* for 10 min at 4°C. Cells were washed twice with 0.05 M potassium phosphate buffer (KPB) at pH 7.0. Final cell resuspension was in KPB, and the cell density was adjusted to 10^9 cells ml^{-1}. A Coulter Counter (model Z; Coulter Electronics, Hialeah, Fla.) was used to measure cell density whenever necessary.

The bacterial chemotactic response towards

mostos was tested by the capillary assay technique of Adler (1) as modified by Hazen et al. (13). After incubation for 1 h at 34°C, the capillary tubes were removed. The sealed end of each capillary tube was broken off, and their contents were washed into dilution vials containing 10 ml of a sodium azide-free isotonic diluting solution (Fisher Scientific Co., Fairlawn, N.J.). Cell counts were made directly from the diluting vial with the Coulter Counter. None of the bacteria showed significant reduction in motility after the incubation period, as indicated by microscopic observations of hanging drop preparations.

Oxygen uptake rates were determined at 34°C with an oxygen electrode (model 53 biological oxygen monitor; Yellow Springs Instrument Co., Yellow Springs, Ohio) connected to a chamber of 3-ml capacity. Oxygen consumption was determined for incubation mixtures containing 1.0 ml of the cell suspension and 2.0 ml of either KPB or the rum slops, as required. The oxygen uptake of cell suspensions was monitored at 1-min intervals. The rate of oxygen assimilation was calculated as the percentage of oxygen removed from a solution initially containing 15 μl of oxygen.

All dilutions of each substrate, the KPB control, and the motility test were tested for differences by using analysis of variance. All counts were transformed with log (x) before analysis, to reduce heteroscedascity as determined by skew and kurtosis. Group means found to be significantly different were further differentiated from each other statistically by using a Student-Newman-Keuls multiple-range test. Any probability less than or equal to 0.05 was considered significant (24).

Differences between cell accumulation due to alterations in the frequency of flagellar beatings (motility) and true chemotactic responses were demonstrated by adding the test substrate to cell suspensions and then measuring the accumulation of bacterial cells into capillary tubes containing KPB. This system was used as a motility control. The mean number of cells drawn into capillary tubes after 1 h of incubation at 34°C was greatest in the motility test for *A. hydrophila* and *E. coli* and in the undiluted mostos for *V. cholerae* and *P. aeruginosa* (Fig. 1). Although the rum distillery waste stimulated motility in *A. hydrophila* and *E. coli* isolates, it did not induce a positive or a negative chemotactic response in *A. hydrophila*. *E. coli* showed a positive chemotactic response to mostos; however, it was significantly lower than the response measured in the motility control (Fig. 1 and Table 1). On the other hand, *P. aeruginosa* and in particular *V. cholerae* showed a strong positive response to artificial chemical gradients of the rum slop. Here, the response of both isolates

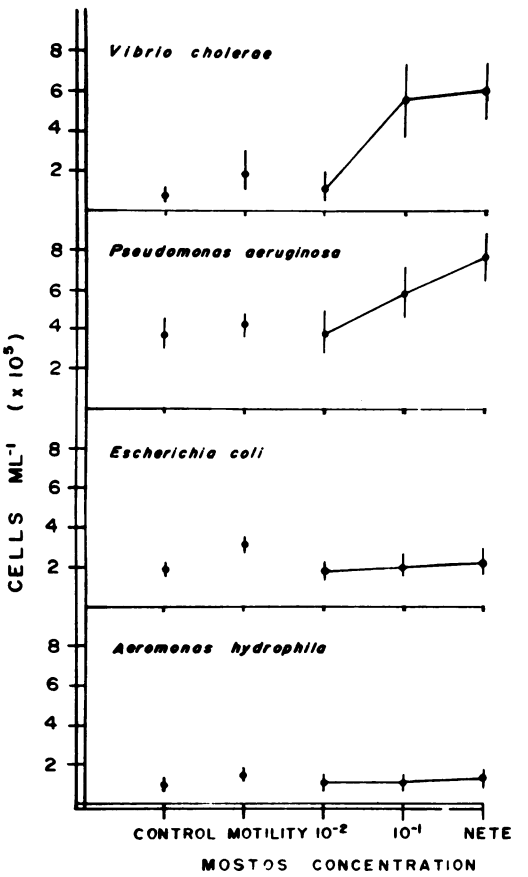

FIG. 1. Chemotactic and motility response to rum distillery effluent for four bacterial isolates (all values are the mean of 10 determinations; bars represent ±1 standard error). Nete, Undiluted sample; dilutions, substrate/final volume ratio.

could not be ascribed to a mere increase in cell motility or to accumulation by random movements. In all cases, the dilution of the test substrate was followed by reduction in cell chemotactic response (Fig. 1 and Table 1).

TABLE 1. Chemotactic index and motility test for isolates by concentration of rum distillery effluent[a]

Species	Motility	Chemotactic index for mostos dilution:		
		Nete	10^{-1}	10^{-2}
A. hydrophila	**1.28**	1.06	0.93	0.96
E. coli	**1.71**	**1.20**	1.06	0.92
P. aeruginosa	1.09	**2.03**	**1.55**	1.01
V. cholerae	**4.69**	**13.74**	**12.54**	**2.40**

[a] Each value is the mean of 10 determinations; standard deviations of the mean were always less than 0.15. Boldfaced values are significant as determined by analysis of variance. Nete, Undiluted sample; dilutions, substrate/final volume ratio.

The oxygen uptake of washed cell suspensions was measured in the presence and absence of mostos. In this way, we tested the ability of each isolate to oxidize the organic matter present in rum distillery wastes. The bacteria used in this study were all facultatively anaerobic, heterotrophic, and motile rods. Since reproduction and swimming in bacteria are energy-requiring processes, it seemed likely that in situ our test organisms might obtain energy and carbon from the oxidation of organic substrates present in the rum distillery effluent. For all the bacterial isolates, the highest respiration rates were measured when mostos was added to cell suspensions (Table 2). However, the oxygen uptake rate of *A. hydrophila* and *E. coli* in the presence of rum slops was significantly lower than that observed for *P. aeruginosa* and *V. cholerae*.

Correlation between chemotactic response and respiration rates (Fig. 1 and Table 2) shows that isolates with a strong positive chemotactic response toward mostos also showed a high oxygen uptake rate when exposed to mostos. An increase in cell motility without a significant chemotactic response was always related to a low oxidation rate of rum slops. The motility index (MI) was determined by comparison of the density of cells accumulated inside capillary tubes containing the test substrate (undiluted) against the number of cells drawn into the motility test. A true chemotactic response would be indicated by MI values greater than one. In the same way, accumulation of cells by random movements and stimulation of flagellar beatings was indicated by MI values lower than one. Thus, *A. hydrophila* and *E. coli* both showed that their accumulation in mostos could only occur by random movements and stimulation of flagellar beatings, whereas *P. aeruginosa* and *V. cholerae* showed ability to oxidize mostos and true chemotaxis toward rum slops (Fig. 2).

The present study demonstrated that bacteria may be observed in elevated densities in the effluent plume as the result of quite different

TABLE 2. Effect of mostos on oxygen uptake of four bacterial isolates[a]

Species	Oxygen uptake (nl ml^{-1})		
	Endogenous	Experimental	Difference
A. hydrophila	150 ± 6	300 ± 6	+150
E. coli	345 ± 20	675 ± 15	+330
P. aeruginosa	660 ± 15	1,620 ± 22	+960
V. cholerae	750 ± 24	2,460 ± 56	+1,710

[a] Each value is the mean of five determinations; standard deviations are indicated for each measurement. Differences among all isolates were significant as determined by analysis of variance. Oxygen uptake was determined for cell suspensions containing 3.3 × 10^8 cells.

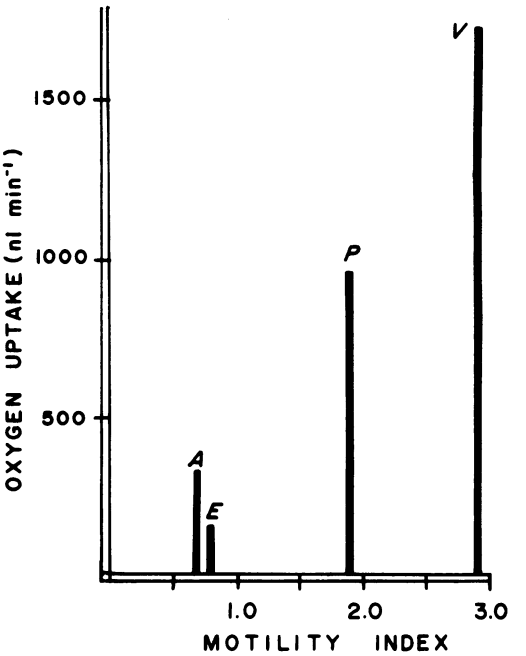

FIG. 2. Oxygen uptake and MI for four bacterial isolates (MI = nete experimental values/motility test for *A. hydrophila* [A], *E. coli* [E], *P. aeruginosa* [P], and *V. cholerae* [V]). Nete, Undiluted sample.

mechanisms. *A. hydrophila*, *E. coli*, and *V. cholerae* all showed significant motility with mostos. Lauffenburger et al. (16) point out in their mathematical model of random motility and bacterial growth that even random motility could promote dispersal from nutrient-poor regions and prevent dispersal from nutrient-rich regions. Only *P. aeruginosa* and *V. cholerae* demonstrated a chemoattraction to mostos which was significantly greater than their motility test for mostos. Thus, chemotaxis may contribute to some extent to the high densities of *P. aeruginosa* and *V. cholerae* in the effluent plume.

Study of the ability of these bacteria to oxidize the rum distillery effluent revealed that only *P. aeruginosa* and *V. cholerae* could significantly oxidize mostos, suggesting that only these bacteria could use undiluted mostos as an energy and carbon source. Although chemotaxis might be involved, it seems likely that the ability to oxidize mostos could alone account for the high densities of *P. aeruginosa* and *V. cholerae* in the effluent plume. It was puzzling to us that *A. hydrophila* and *E. coli* were in high densities and able to survive and grow in the effluent in situ as seen by our diffusion chamber studies (2; A. J. Lopez-Torres, M.S. thesis). It is possible that chemotaxis was being blocked in *A. hydrophila* and *E. coli* by some hydrocarbon fraction of the

mostos. Chet and Mitchell (3) reported that bacterial chemotaxis and hence decomposition could be blocked by petroleum hydrocarbons in marine systems. However, we knew that *A. hydrophila* has been found ubiquitously in the United States and other countries in stressed aquatic environments such as sewage, pulp mill, and nitrogen fertilizer factory effluents and hyperthermal and hypersaline waters (5, 8, 9, 11, 12, 14, 15, 17, 18). In these studies, densities of *A. hydrophila* were closely correlated with chlorophyll *a* concentrations. Indeed, densities of *A. hydrophila* in a southeastern United States estuary fit a mathematical model which relied upon chlorophyll *a* concentrations (10). Biamon and Hazen (2) also found a strong correlation between the concentration of chlorophyll *a* and density of *A. hydrophila* and the density of fecal coliforms in the rum distillery effluent. Thus, nutrients leaking from algae flourishing in the rum distillery effluent plume may support bacterial growth and may even attract bacteria from surrounding areas. This hypothesis is supported by preliminary data on bacterial chemotactic response to algal extracts. The latter have been prepared from *Ulva lactuca* and *Gracilaria foliifera* isolates, collected at the effluent outfall. *A. hydrophila* shows a significant positive chemotactic response (chemotactic index = 1.52) toward these extracts (Fuentes, unpublished data). Close interactions between bacteria and algae have been implicated during algal blooms (10, 20) and for *P. aeruginosa* (6). The exact role of bacteria-algae interactions in the effluent plume remains to be determined.

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