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Incidence of Plasmids in Marine *Vibrio* spp. Isolated from an Oil Field in the Northwestern Gulf of Mexico

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Presumptive marine *Vibrio* spp. were collected from an operational oil field and control site located in the northwestern Gulf of Mexico. Of 440 isolates analyzed for the presence of extrachromosomal deoxyribonucleic acid elements or plasmids by using the cleared lysate and agarose gel techniques, 31% showed distinct plasmid bands on agarose gels. A majority of the plasmids detected were estimated to have molecular masses of 10×10^6 or less. Multiple plasmids were observed in approximately half of the plasmid-containing strains. A number of isolates contained plasmids with similar banding and mobility patterns. The oil field area had noticeably more plasmid-containing strains (35 versus 23% in the control site) and a greater number of plasmids per plasmid-containing strain (an average of 2.5 plasmids, versus 1.5 in the control site). Oil field discharges might have resulted in increased plasmid incidence and diversity.

A majority of the survey work examining bacteria for the presence of extrachromosomal deoxyribonucleic acid (DNA) elements has been restricted either to medically important bacteria (1, 5) or to plant pathogens (10). There have been some ecological studies to determine the incidence of plasmids in naturally occurring nonpathogenic terrestrial bacteria (14, 18, 22), but few reports have dealt with plasmid distribution in marine bacteria (9).

The marine *Vibrio* spp., also referred to as *Beneckea* spp. by other investigators (3), are a group of gram-negative, facultatively anaerobic rod-shaped bacteria (20) which occur throughout the world in bays, estuaries, and marine waters. Members of this group are important as human (19) and animal (8) pathogens. In many areas, *Vibrio* spp. are seasonally a significant proportion of the total population of bacteria found in the ocean's water column (13). They are closely related taxonomically to the enteric bacteria and in the marine environment fill ecological niches that in the terrestrial environment are filled by enteric bacteria (3).

The marine environment has been characterized as nutritionally starved (4). Although alternate views exist (9, 15), some (21) believe that the effects of an energy-limiting situation will negatively influence the maintenance of nonessential factors such as plasmids, since a balance between genetic flexibility and the metabolic load must be maintained. Isolates studied in this report were collected from locations within an operational gas and oil field and a control site located outside the production area. The producing gas and oil field has been established for a number of years and releases as a result of its operation chronic low levels of pollution in the form of hydrocarbons and sulfur (17).

This study will attempt to determine the incidence of plasmid DNA in *Vibrio* spp. and to determine whether this incidence follows the pattern reported for resistant plasmids in enteric bacteria (17, 18). Furthermore, incidence data between the control site and the chronically polluted platform site will be compared to determine the effect, if any, of the presence of the platform pollutants on the bacterial plasmid incidence.

MATERIALS AND METHODS

Bacterial reference strains. All reference organisms utilized for the production of plasmid standards are strains of *Escherichia coli*. Those containing the plasmids ColE1 (4.2 megadaltons [Mdal]), R6-5 (61 Mdal), RP4 (34 Mdal), and R6-K (24.7 Mdal) were provided by D. Zink (Texas A & M University, College Station). A strain containing plasmid Pm88 (1.7 Mdal) was obtained from G. Thorn (Tufts University, Boston, Mass.).

Isolation and cultivation of presumptive marine *Vibrio* spp. Marine *Vibrio* spp. were collected during four quarterly (spring, summer, fall, and winter) cruises to an operational gas and oil field located ca. 49.6 km south-southeast of Galveston, Tex., in about 20 m of water. Three of the sampling sites were located within the oil field area near a production platform. Sites 1, 2, and 3 were located at 30, 60, and 90 m, respectively, south of the platform. The control station was located ca. 8 km north of the production area. Water currents run northeast in summer and reverse to the southwest during October to April (2). The control site closely resembled the oil field stations in geological stratum, water depth, and physical param-

eters (water temperature, pH, and salinity). Water samples were taken from 3 levels (surface, mid-water, bottom) to a depth of about 18 m. Sterile glass water bottles were used to collect surface water, and a Niskin sampler (General Oceanics Inc., Miami, Fla.) was used for the other samples. Water samples were plated on thiosulfate-citrate-bile salts-sucrose (TCBS) medium (BBL Microbiology Systems, Cockeysville, Md.) aboard ship and incubated at ambient temperature anerobically. Isolates growing on the TCBS medium were tested for sensitivity to Vibriostat 0/129 (BDH Chemical Ltd., Poole, England). Their sensitivity was consistent with their being presumptive *Vibrio* spp. Characterization of two random isolates from each sampling location on API 20 strips (Analytab Products, Plainview, N.Y.) was also consistent with the isolates being *Vibrio* spp. The isolates were maintained on agar slants consisting of marine medium MSWYE, the preparation of which has been described previously (7).

Cell lysis and plasmid isolation. Bacteria were grown in 40 ml of MSWYE medium to between 5×10^7 and 5×10^8 bacteria ml^{-1}. Cells were lysed by using either the technique of Hansen and Olsen (12) or a modified version of the cleared lysate technique of Clewell and Helinski (6). Modifications of the latter included washing bacterial cells in 3% NaCl and 10^{-3} M ethylenediaminetetraacetic acid and reducing the amount of Brij solution used to lyse the cells from 50 to 20%.

Agarose gel electrophoresis. Ethanol-precipitated DNA obtained from the lysate techniques was dissolved in 0.1 ml of TES buffer consisting of 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid, and 20 mM tris(hydroxymethyl)aminomethane at a pH of 8.0. One-half of this sample was diluted to a volume of 0.3 ml with TES buffer and examined on a Gilford spectrophotometer 250 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) to determine DNA concentrations. Approximately 40 μg of DNA from each isolate was examined for plasmid DNA by using the agarose gel electrophoretic technique described by Meyers et al. (16). The average molecular masses of *Vibrio* spp. plasmids were determined by comparing them with *Escherichia coli* plasmid standards.

Hydrocarbon utilization. Each isolate was tested for hydrocarbon utilization by inoculating it into an artificial seawater solution containing filter-sterilized oil obtained from the oil field. The artificial seawater-oil solution consisted of 0.4 M NaCl, 0.028 M $MgSO_4 \cdot 7H_2O$, 0.01 M KCl, 0.001 M KNO_3, 0.007 M $(NH_4)_2SO_4$, 0.007 M KH_2PO_4, 0.006 M K_2HPO_4 and 0.1 ml of oil, pH 7.6. Utilization of hydrocarbons was indicated by visible turbidity at the oil-artificial seawater interface.

Resistance to heavy metals. Each isolate was tested for resistance to lead (230 ppm [230 mg/liter]), mercury (8 ppm), and zinc (110 ppm). Isolates were spotted with sterile cotton swabs on agar plates containing MSWYE medium and the appropriate salt form of each metal. Resistance was determined by the presence or lack of growth.

RESULTS

A total of 473 presumptive marine *Vibrio* spp. isolated from the Gulf of Mexico were examined for the presence of extrachromosomal DNA elements. Of these isolates, 440 gave good lysates and 33 were eliminated from the study because the screening procedures were ineffective in lysing them. Table 1 lists the number of isolates for which analyses were made and the incidence of plasmids in samples taken from each station during four quarterly cruises. During the summer and fall cruises, three samples from each sampling site produced an average of 170 and 120 presumptive *Vibrio* spp. per ml of seawater, respectively. During the winter and spring samplings, as much as a fivefold reduction in the average number of isolates was observed. Of the 440 isolates examined, 137, or 31%, showed distinct plasmid bands on agarose gels. A breakdown of the totals by site showed that a higher percentage of plasmid-containing *Vibrio* spp. were isolated from the oil field area (35%) than from the control station (23%). Control station isolates taken during the winter and spring cruises showed higher plasmid incidence than did isolates from the oil field site; however, low numbers of organisms isolated during these periods may have led to inaccurate results.

A number of plasmid-containing *Vibrio* spp. appeared to be similar strains with identical numbers of plasmid bands and mobility patterns appearing on photographs of gels. Figure 1, which shows the photograph of an agarose gel, demonstrates this situation. Lanes A, B, C, D, E, and F contained lysates prepared from six different isolates taken from the same site on the same cruise. Physiological tests, including amino acid metabolism, sugar metabolism, and antibiotic resistance patterns, performed on these groups of isolates further confirmed that the strains were identical (Hada and Sizemore, manuscript in preparation). When strains with identical numbers of plasmids and mobility patterns were scored as a single plasmid-containing strain, the resulting 397 isolates had 96, or 24%, plasmid-containing strains. Once again, under this subjective system of scoring, the oil field

TABLE 1. *Incidence of Vibrio spp. and plasmid-containing Vibrio spp. for the gas and oil field stations and control site during four quarterly cruises*

Season	Oil field stations		Control stations	
	No. of *Vibrio* spp.	% *Vibrio* spp. with plasmids	No. of *Vibrio* spp.	% *Vibrio* spp. with plasmids
Fall	58	45	90	21
Winter	42	30	3	66
Spring	35	23	6	33
Summer	168	35	38	21

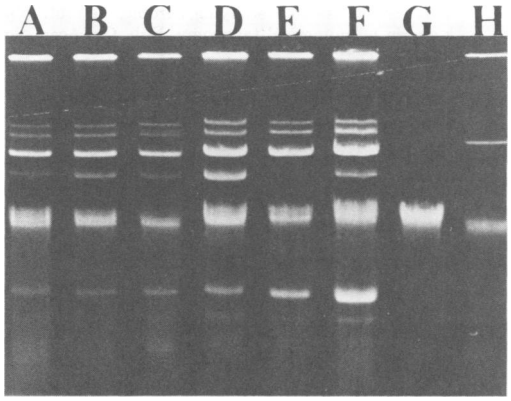

FIG. 1. *An agarose gel containing lysates prepared from Vibrio spp. isolated from the northwestern Gulf of Mexico. Lanes A, B, C, D, E, F, and H contain lysates from seven different isolates taken from the same site on the same cruise. Lane G contains a chromosomal marker prepared from E. coli EC 185.*

sites showed higher percentages (27%) of strains containing plasmids than did the control site (18%).

Size classes of the plasmids were estimated by comparing relative mobilities of unknown plasmids with those of the plasmid standards. Over half of all plasmids detected fell into the range of 0 to 12 Mdal. The vast majority of plasmids within that range were estimated to have a molecular mass of either 0 to 12 or 9 to 11 Mdal. A substantial number of plasmids were detected in the size range between 10 and 80 Mdal. Very few plasmids were estimated to have a mass greater than 80 Mdal, and none larger than 100 Mdal were observed.

Table 2 lists the plasmid multiplicity of plasmid-containing strains. Approximately half of all plasmid-containing strains possessed a single plasmid. However, the occurrence of multiple plasmid bands ranging from two to seven species in individual isolates was not an uncommon event. Seven plasmid bands in an individual isolate was the maximum number detected. An examination of plasmid multiplicity (Table 2) shows a difference in the number of plasmids for plasmid-containing isolates in the control site compared with the oil field stations. Seventy-one percent of the plasmid-containing strains from the control station showed single plasmid bands. In contrast, the majority (58%) of the plasmid-containing isolates from the oil field sampling sites possessed multiple plasmids. Some of the multiple plasmids detected may be single species linked to form dimers and trimers of each other. In a few cases, estimation of molecular weight of multiple plasmids was found to be double or triple the mass of a small plasmid band in the same strain.

Plasmid-containing strains were tested for both hydrocarbon utilization and heavy metal resistance since these factors are the most apparent influences that could affect plasmid incidence and diversity. None of the plasmid-containing strains demonstrated visible growth in the artificial seawater-oil medium. Although some of the plasmid-containing strains were resistant to individual metals tested, percentages of total resistant strains were not noticeably greater than plasmid incidence among isolates from the oil field stations.

DISCUSSION

An extensive survey of 440 presumptive marine *Vibrio* spp. for the incidence of extrachromosomal DNA elements has indicated that plasmids occur frequently in this group of organisms. No apparent trend by season in the percentage of strains with detectable plasmids was observed. In general, marine *Vibrio* spp. carry plasmids with small molecular masses. The majority of *Vibrio* spp. plasmids were 10 Mdal or less, and none greater than 100 Mdal was observed. Finally, the occurrence of multiple plasmids in marine *Vibrio* spp. is a relatively common event. The reason for this phenomenon is not known.

The only other survey of plasmids in marine *Vibrio* spp. reported a plasmid incidence of 25% (11), similar to the 23% in our control station isolates. The 23% incidence in the control station isolates is slightly greater than the 10 to 20% incidence of R factors reported in enteric bacteria (21, 22). The differences in the average incidence and multiplicity of plasmids between isolates from the control site and the oil field locations are an ecologically interesting discovery. The oil field stations are located in an area which has a greater influx and variety of nutrients and toxic materials than the control station because of the activities associated with an operational oil field (17). Although hydrocarbon utilization and resistances to heavy metals are not apparent important influences, other enrich-

TABLE 2. *Plasmid multiplicity of the plasmid-containing strains*

Plasmid bands per strain	Percentage of plasmid-containing strains	
	Oil field isolates	Control station isolates
1	39.6	71
2	24.5	16
3	16.0	0
4	3.8	13
5	2.8	0
6	7.5	0
7	5.7	0

ing materials may create a situation which selects for the higher incidence of plasmid-containing strains and plasmid types within individual strains among isolates from the oil field site. This might also be true for the differences in plasmid multiplicity.

In the marine environment, enrichment processes may be an influence in the maintenance of plasmids in *Vibrio* spp. If this is true, plasmid multiplicity could be used as an indicator of some types of water pollution. In addition, plasmids in marine bacteria must be studied to understand ecological implications of adaptations to situations such as the establishment of offshore oil fields. The next step in this study is to determine the function of plasmids in marine *Vibrio* spp. This will help establish the ecological role of extrachromosomal DNA elements in this group of organisms.

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