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Bacterial Plasmids in Antarctic Natural Microbial Assemblages

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Samples of psychrophilic and psychrotrophic bacteria were collected from sea ice, seawater, sediments, and benthic or ice-associated animals in McMurdo Sound, Antarctica. A total of 155 strains were isolated and tested for the presence of plasmids by DNA agarose gel electrophoresis. Thirty-one percent of the isolates carried at least one kind of plasmid. Bacterial isolates taken from sediments showed the highest plasmid incidence (42%), and isolates from seawater showed the lowest plasmid incidence (20%). Plasmids were significantly more frequent in the strains which had been first isolated from low-nutrient media (46%) than in the strains which had been isolated from high-nutrient media (25%). Multiple forms of plasmids were observed in two-thirds of the plasmid-carrying strains. A majority of the plasmids detected were estimated to have a mass of 10 megadaltons or less. Among 48 plasmid-carrying strains, 7 showed antibiotic resistance. It is concluded that bacterial plasmids are ubiquitous in natural microbial assemblages of the pristine marine ecosystem of Antarctica.

There have been some ecological studies to determine the incidence of plasmids in natural populations of terrestrial bacteria (7, 11), but few reports have dealt with the incidence of plasmids in natural populations of aquatic environments. Hada and Sizemore (6) examined the plasmid incidence of marine *Vibrio* species in the Gulf of Mexico and found the occurrence of plasmids to be higher in oil field regions (35%) than in the control area (23%). Glassman and McNicol (3) found that 46% of the estuarine bacteria from the sediment and water column in Chesapeake Bay carry plasmids. They also found that bacteria from clean sites tend to carry small plasmids (3 megadaltons [Mdal]), whereas those from more polluted sites generally carry multiple plasmids which are larger than 30 Mdal. Simon et al. (15) isolated 58 marine luminous bacteria mainly from the Mediterranean and Red Seas, and found that 43% of the bacteria carry plasmids. A survey for plasmids in the freshwater environment of the South Wales River reported a plasmid incidence of 10% in unpolluted sites versus 15% in polluted ones (1).

No reports have dealt with the occurrence of plasmids in natural bacterial assemblages of polar regions. In fact, few psychrophilic and psychrotrophic bacteria have been isolated from these areas (12). Recently, however, Sullivan and his colleagues (17, 18; S. McGratt-Grossi, S. T. Kottmeier, and C. W. Sullivan, Microbial. Ecol., in press) have reported the presence of diverse, abundant microbial communities associated with annual sea ice which seasonally covers the surface of polar oceans. These organisms live and grow in a constantly cold environment whose temperatures seldom vary from the −1.9°C ambient by more than a degree. Although physically an extreme environment, the Southern Ocean regions have been little influenced by human activities. The present study will attempt to determine the frequency of plasmid appearance in psychrotrophic and psychrophilic bacteria collected from various Antarctic sources, such as sea ice, seawater, sediments, and animals. Our data will provide base-line information about plasmid frequency in natural microbial populations, since one of the areas studied has been unperturbed by activities of humans. This is the first report of a bacterial plasmid study in natural microbial assemblages of the pristine marine ecosystem of Antarctica.

MATERIALS AND METHODS

Collection of samples. Samples were obtained in McMurdo Sound, Antarctica, at Cape Armitage (station A) from November through December 1980, and in New Harbor (station B) from October 1981 through January 1982 (Fig. 1). Sea ice samples were collected from the bottom 20 cm of the ice by using standard Snow Ice Permafrost Research Establishment ice augers or from the ice-seawater interface by scuba divers using a sterile syringe. Water samples were collected from the water column 1 m below the sea ice with an ethanol-cleaned Kemmer sampling device. Sediment samples were collected from the bottom of the water column by scuba divers using a sterile syringe. Zooplankton samples such as copepods, amphipods, and isopods were collected in a 300-m net tow near station A. Benthic animals such as yellow and red sponges, gorgonians, and a pycnogonid were taken from the sediment or water column. All samples were kept frozen at −20°C until bacterial isolation was carried out.

Bacterial isolation. The frozen samples were thawed at 0°C just before bacterial isolation. Zooplankton, the guts of isopods, and pycnogonid were then homogenized. Serially diluted samples of melted ice, seawater, sediment interstitial water, and homogenate of animal samples were next spread onto agar plates. For the isolation of bacteria from station A, the following four media were employed: (i) 2216E medium (14); (ii) diatom filtrate medium, prepared by adding 100 ml of filter-sterilized supernatant of five Antarctic diatom culture fluids (1×10^6 to 2×10^6 cells per ml) to 900 ml of sterilized seawater; (iii) 1,000-fold-diluted Difco 2216E medium with aged seawater; and (iv) unamended seawater medium. For the isolation of bacteria from station B, the following three media were used: (i) Difco Actinomycete isolation agar, supplemented with glucose (5 g/liter); (ii) malt extract medium (13); and (iii) f/2 agar medium (2). Each sample was spread onto these three or four media, and the plates were incubated in darkness at −1 to +1°C for 2 to 12 weeks in a Psychrotherm low-temperature incubator. Morphologically different colonies were picked from the plates. The 2216E

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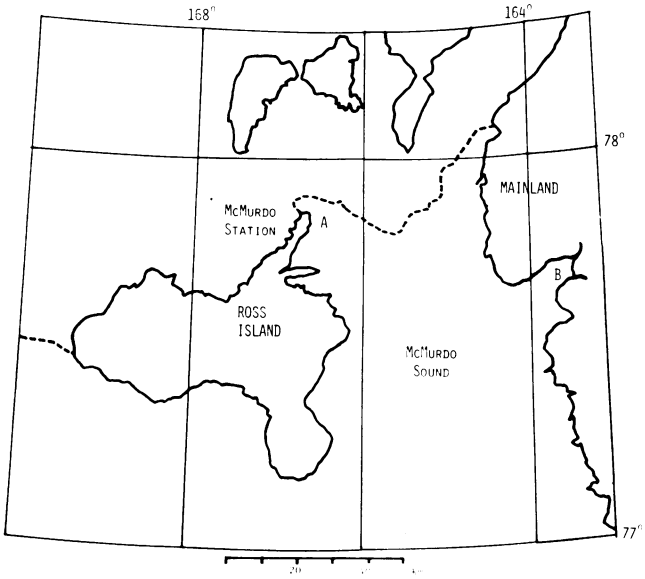

FIG. 1. Sampling stations in McMurdo Sound, Antarctica. The A denotes station A at Cape Armitage and B denotes station B at New Harbor.

medium was used for purification and maintenance of the Antarctic bacteria.

Cell lysis and plasmid isolation. Bacteria were grown in 60 ml of 2216E medium to a concentration of 5×10^8 cells per ml. The method of Guerry et al. (5) was modified to lyse the cells of marine bacteria. One such modification involved washing the bacterial cells once with 3% NaCl in 50 mM Tris-hydrochloride buffer (pH 7.6) before lysis. The clear lysate containing plasmid DNA was precipitated with ethanol and centrifuged. The precipitated DNA was dissolved in 20 μl of sterilized E buffer containing 40 mM Tris-acetate and 2 mM sodium-EDTA at pH 7.9. Ten microliters of the DNA sample was treated with 2 μl of RNase A (bovine pancrease, Worthingon Diagnostics, Freehold, N.J.) in 200 mM potassium acetate (pH 4.8) at 30°C for 30 min.

DNA agarose gel electrophoresis. The reaction mixture described above (12 μl) was mixed with 3 μl of a dye solution consisting of 33% glycerol, 7% sodium dodecyl sulfate, and 0.07% bromophenol blue. The solution was examined by 0.8% agarose gel electrophoresis, and the plasmid DNA was visualized by the technique described by Meyers et al. (10). The molecular weights of the plasmids were determined by comparing them with four standard plasmid DNA markers (New England Nuclear Corp., Boston, Mass.) including pUK2 (1.48 Mdal), pBR322 (2.88 Mdal), pBR313 (6.4 Mdal), and pJC74 (11.6 Mdal).

Antibiotic resistance test. Yeast-peptone medium was used as the basal medium to determine the antibiotic sensitivity of the Antarctic bacteria. This medium consisted of 0.5% Bacto-Peptone, 0.1% yeast extract (Difco Laboratories, Detroit, Mich.), 2.3% NaCl, 0.7% $MgSO_4$, and 0.08% KCl in distilled water, and was adjusted to pH 7.2. Antibiotics and their concentrations were as follows: ampicillin (Am), 50 μg/ml; tetracycline, 250 μg/ml; nalidixic acid, 50 μg/ml; chloramphenicol, 10 μg/ml; gentamicin sulfate, 10 μg/ml. MICs of the antibiotics were determined by spreading *Escherichia coli* C600 on yeast-peptone medium agar plates. The susceptibility to drug was tested by spotting a drop of the cultured bacteria onto plates with or without antibiotics. Bacteria were classified as antibiotic resistant only if they grew equally well on both plates.

Mating and transformation experiments. Plasmid-carrying strains with Am drug resistance were chosen for mating and transformation experiments. For the mating experiment, cells of *E. coli* HS594 (C600 *hsr hsm dapD*) were used as recipient cells and mating was attempted at 2, 15, and 25°C by the method of Sizemore and Colwell (16). Transformation experiments involving Am resistance markers in Antarctic bacteria to *E. coli* was performed by the method of Mandel and Higa (8). Plasmid DNA of the Antarctic bacteria was purified by either ethidium bromide-cesium chloride density gradient or agarose gel electrophoretic separation (9). This was followed by Eluptip-d column treatment according to the instructions supplied by Schleicher & Schuell, Inc., (Keene, N.H.). The transformation mixture and selected agar plates were incubated at 20°C instead of 37°C because of the psychrotrophic character of the bacteria.

Statistical treatment of data. A statistical test to determine the plasmid-carrying frequencies in two different populations of bacteria isolated from low- and high-nutrient media was performed. Since sample sizes were large, the following approximation could be used (4):

$$(P_1 - P_2) \Big/ \sqrt{\left[\pi\,(1-\pi)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)\right]}$$

where n_1 and n_2 are the numbers of isolates on low- and high-nutrient media, respectively. P_1 and P_2 are the proportions of bacteria with plasmids on low- and high-nutrient media, respectively.

Pi (π) is the overall proportion of bacteria with plasmids at the two sites. This test statistic has the standard normal distribution under the null hypothesis that the proportions of bacteria with plasmids on two media are equal.

RESULTS AND DISCUSSION

A total of 155 psychrophilic and psychrotrophic bacteria were isolated from 30 samples collected from Antarctic sea ice, seawater, sediments, and benthic or ice-associated animals. Seven different media were used for isolation of bacteria to permit collection of a wide range of isolates from natural populations. Psychrophilic bacteria are defined by their ability to grow at 0°C but not 18°C. Psychrotrophic bacteria are those able to grow at 0 and 18°C or higher. Among 155 strains isolated, 32 strains (21%) were psychrophilic bacteria and 123 strains (79%) were psychrotrophic bacteria.

Of the 155 strains tested for the presence of plasmids, 48 (31%) showed distinct plasmid bands on DNA agarose gel (Table 1). Bacterial isolates taken from sediment had the

TABLE 1. Frequency of plasmid-carrying strains isolated from different sample sources

Sampling station[a]	Source of strain	No. of bacteria tested	No. of strains with plasmids	% of strains with plasmids
A	Sea ice	79	24	30
	Sediment	24	10	42
	Seawater	25	5	20
	Animal[b]	9	3	33
B	Sea ice	18	6	33

[a] See Fig. 1.
[b] Pycnogonid, amphipod, and red sponge.

highest plasmid incidence (42%), and those from seawater had the lowest plasmid incidence (20%). Bacterial isolates obtained from two distant station locations exhibited almost the same frequency of plasmid incidence. A comparison of plasmid-carrying frequencies between psychrophilic and psychrotrophic strains indicated that the percentage of psychrotrophic bacteria carrying plasmids was twice that of psychrophilic bacteria. We tested 32 strains of psychrophilic bacteria and found that five carried plasmids (16%), whereas 123 psychrotrophic strains tested revealed 43 with plasmids (35%). We have no explanation for this difference at present.

Because a number of the plasmid-carrying bacteria isolated from the same set of given samples (Table 1) were found to possess identical banding patterns on agarose gel electrophoresis, we suspected that they were actually the same strains. A limited number of taxonomic and physiological tests, such as Gram reaction, morphology, motility, growth temperature, antibiotic resistance patterns, phosphatase activity, and vitamin B_{12} production and utilization provided further evidence that the strains were identical. When these strains were scored as a single plasmid-carrying strain, the resulting 149 isolates were estimated to contain 42 plasmid-carrying varieties, for a frequency of 28%. Other searches for plasmids in natural populations of marine bacteria have reported the following frequencies: 23% in an unpolluted site of the Gulf of Mexico (6); 46% in Chesapeake Bay (3); and 43% in marine luminous bacteria in the Mediterranean and Red Seas (15). Our estimated plasmid frequency of 28% in Antarctic bacteria is thus within the range of values cited by these reports (3, 6, 15). However, we believe that the actual frequency of plasmid occurrence in the Antarctic bacteria may be higher than our estimated 28% value for the following reasons. First, the technique we used could not permit us to detect plasmids of greater than 65 Mdal (5). Second, bands of chromosomal DNA which can appear on an agarose gel may obscure plasmid bands. Third, one particular lysing protocol may not be suitable for demonstrating the presence of plasmids in every bacterial species examined here. Finally, our technique would not allow detection of those plasmids that are integrated within the host chromosome.

Frequency of plasmid incidence differed significantly depending on the medium used for the initial bacterial isolation (Table 2). Plasmids were more frequent in the strains which had been first isolated from low-nutrient media (46%) as compared with the high-nutrient isolates (25%). This difference was statistically tested (4) and found to be significant at the 1% level. It is interesting that the frequency of plasmid occurrence is influenced by the media used for the first isolation of bacteria. Although an explanation for this phenomenon is not available at present, one might speculate as to a possible cause. Plasmids may encode traits which permit higher growth rates under conditions in which nutrients are at very low concentrations, such as those in unamended seawater and f/2 medium. If this were true, then bacteria which harbor plasmids would have a selective advantage, and these strains would be expressed sooner as colonies than would plasmidless strains. We observed that unamended seawater and f/2 medium, which contained no added organic substances, showed the highest plasmid incidence (50%). Likewise, strains harboring plasmids may be at a selective disadvantage under conditions (high-nutrient media) in which the selective pressure for plasmid-borne functions is relaxed. Although this hypothesis is testable in the laboratory, it was beyond the scope of the present study. These media may be useful in future studies for selecting plasmid-carrying marine bacteria from natural populations.

TABLE 2. Frequency of plasmid-carrying strains isolated on different media

Nutrient condition	Medium used for first isolation of bacteria	Sampling station[a]	No. of strains tested	% of strains with plasmids
High	2216E	A	92	24
	Diatom filtrate medium	A	10	2
	MYE or AIM[b]	B	12	3
Low	Diluted 2216E	A	17	7
	Seawater	A	18	9
	f/2	B	6	3

[a] See Fig. 1.
[b] MYE, Malt extract; AIM, Actinomycete isolation agar supplemented with glucose.

TABLE 3. Molecular weights of plasmids isolated from Antarctic strains

Plasmid size (Mdal)	No. of plasmids
>10	38
5–10	22
2.5–5	23
<2.5	22
Unknown	9

Size classes of the plasmids were estimated by comparing the relative migration of unknown plasmids with that of four plasmid molecular weight markers. Sixty-four percent of the plasmids had a molecular weight smaller than 10 Mdal (Table 3). Figure 2 shows a typical electrophoretic separation of the plasmids. It was prepared from eight isolates exhibiting a wide range of plasmid molecular weights. Lane 2 contained the largest plasmid detected in this study, and lane 9 showed the smallest plasmid detected. Lanes 4 and 6 contained small plasmids of 1.5 Mdal. It is noteworthy that plasmids having a molecular weight smaller than 2.5 Mdal were not uncommon, and 21% of the plasmids detected fell into a class of molecular weights less than 2.5 Mdal. The smallest plasmid detected had a molecular weight of ca. 1.0 Mdal (ca. 1,500 base pairs) (lane 9 in Fig. 2). This may be the smallest plasmid so far reported in natural environments. Our results agree with those of Hada and Sizemore (6), who found the majority of plasmids in marine planktonic vibrios to be 10 Mdal or smaller. Glassman and McNicol (3) also found a preponderance of small plasmids in marine isolates from clean sites but larger plasmids (30 Mdal) in isolates from a more polluted site.

Two-thirds (67%) of the plasmid-carrying strains showed multiple plasmid bands ranging from 1 to 10 molecular weight species in individual isolates. Ten plasmid bands from HK192 was the maximum number detected (lane 9 in Fig. 2). Since one plasmid can give three bands (closed circular, open circular, and linear DNAs) and the technique used (5) in this study detects replicative intermediate forms of plasmids as well as multimers, we are not certain how many separate plasmids these multiple bands represent.

Seven antibiotic-resistant strains were found among 48 plasmid-carrying strains, whereas only 2 of the 107 non-plasmid-carrying strains were found to be antibiotic resistant. The seven antibiotic-resistant strains isolated from plasmid-carrying bacteria had several common properties. (i) All seven strains were taken from the sediments at three different locations; (ii) all seven strains were resistant to Am; (iii) each strain had a single small plasmid of molecular weight 1.5 Mdal (lanes 4 and 6 in Fig. 2 show the 1.5-Mdal

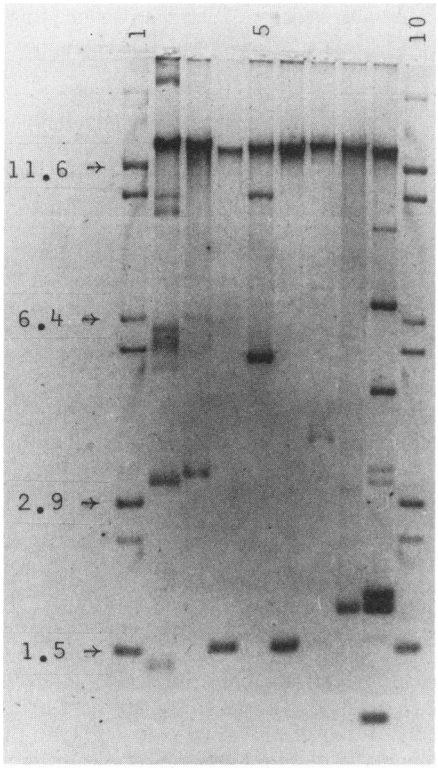

FIG. 2. Identification of plasmids by DNA agarose gel stained with ethidium bromide. Gel electrophoresis was carried out in TBE buffer, pH 8.4 (89 mM Trizma base, 89 mM boric acid, and 2.5 mM disodium-EDTA) at 1.5 V/cm for 20 h. Migration distances of the molecular markers are shown in lanes 1 and 10. Lanes 2 to 9 are plasmids from strains HK1, 42, 60, 90, 108, 114, 117, and 192, respectively.

plasmids whose hosts were resistant to Am). These common properties indicate that these bacteria may share a common milieu and also a common mode of developing antibiotic resistance.

The demonstration of higher incidence of antibiotic resistance in plasmid-carrying strains suggests the possible existence of plasmid-borne resistances. But since non-plasmid-carrying bacteria also showed drug resistance, more evidence is needed before this can be verified. Mating and transformation experiments of the Am resistance marker to *E. coli* were conducted to test the idea; however, both experiments were unsuccessful, leaving the question unanswered.

In conclusion, our study suggests that plasmids are ubiquitous in natural microbial populations of the pristine marine ecosystem of Antarctica. As such, these data provide preliminary information to microbial ecologists who are interested in examining the functions and evolution of plasmids in natural environments which are essentially free of selective pressures due to anthropogenic materials. Our data also indicate that selection of media is an important factor in estimating the frequency of plasmids in natural microbial populations.

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