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Plasmids Isolated from Marine Sediment Microbial Communities Contain Replication and Incompatibility Regions Unrelated to Those of Known Plasmid Groups

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Two hundred ninety-seven bacteria carrying plasmids that range in size from 5 to 250 kb were identified from more than 1,000 aerobic heterotrophic bacteria isolated from coastal California marine sediments. While some isolates contained numerous (three to five) small (5- to 10-kb) plasmids, the majority of the natural isolates typically contained one large (40- to 100-kb) plasmid. By the method of plasmid isolation used in this study, the frequency of plasmid incidence ranged from 24 to 28% depending on the samples examined. Diversity of the plasmids occurring in the marine sediment bacterial populations was examined at the molecular level by hybridization with 14 different DNA probes specific for the incompatibility and replication (*inc*/*rep*) regions of a number of well-characterized plasmid incompatibility groups (repB/O, FIA, FII, FIB, HI1, HI2, I1, L/M, X, N, P, Q, W, and U). Interestingly, we found no DNA homology between the plasmids isolated from the culturable bacterial population of marine sediments and the replicon probes specific for numerous incompatibility groups developed by Couturier et al. (M. F. Couturier, F. Bex, P. L. Bergquist, and W. K. Maas, Microbiol. Rev. 52:375–395, 1988). Our findings suggest that plasmids in marine sediment microbial communities contain novel, as-yet-uncharacterized, incompatibility and replication regions and that the present replicon typing system, based primarily on plasmids derived from clinical isolates, may not be representative of the plasmid diversity occurring in some marine environments. Since the vast majority of marine bacteria are not culturable under laboratory conditions, we also screened microbial community DNA for the presence of broad- and narrow-host-range plasmid replication sequences. Although the replication origin of the conjugally promiscuous broad-host-range plasmid RK2 (*incP*) was not detectable in any of the plasmid-containing culturable marine isolates, DNA extracted from the microbial community and amplified by PCR yielded a positive signal for RK2 *oriV* replication sequences. The strength of the signal suggests the presence of a low level of the *incP* replicon within the marine microbial community. In contrast, replication sequences specific for the narrow-host-range plasmid F were not detectable in DNA extracted from marine sediment microbial communities. With the possible exception of mercuric chloride, phenotypic analysis of the 297 plasmid-bearing isolates did not demonstrate a correlation between plasmid content and antibiotic or heavy metal resistance traits.

Numerous studies have reported on the incidence of bacterial plasmids in marine sediments and estuarine and pelagic ecosystems (5, 24, 29, 49, 56). Plasmid-encoded genes represent a pool of mobile DNA that contributes significantly to the genetic adaptation of natural microbial communities. An example of such plasmid-mediated adaptation has been reported previously for microbial populations during periods of pollutional stress when the frequency of catabolic plasmids increased by as much as 2- to 10-fold (11, 23, 37). One explanation for the increased plasmid frequency is the dissemination of plasmid-encoded catabolic genes, through either their self-transmission or mobilization within the microbial community. However, there remains a general lack of information, particularly at the molecular level, regarding plasmids present in natural bacterial assemblages. Thus, to better understand bacterial gene flux in natural systems, information on the distribution and diversity of plasmids within microbial communities is necessary.

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To date, two general approaches for examining plasmid abundance and distribution in natural microbial communities have been described. One method, endogenous isolation, requires the initial isolation of bacteria to test for the presence of plasmids. The second approach, exogenous isolation, does not require the isolation of the plasmid-bearing host bacterium but instead relies primarily on the ability of natural plasmids to transfer to a selected recipient or to mobilize a nonconjugative plasmid (20, 25a). Presently, most indigenous plasmids, particularly from marine ecosystems, have been only particularly characterized with respect to conjugation proficiency, replication, and incompatibility groups. In addition, a number of studies have reported the inability to detect selectable traits encoded by plasmids isolated from environmental sources (5, 8, 24, 48). Previously, a TOL plasmid (i.e., encoding toluene degradation), originally isolated from *Pseudomonas putida*, has been demonstrated to have broad-host-range capabilities based on its conjugal transfer to and maintenance in *Escherichia coli* (6). More recently, approximately two-thirds of the plasmids isolated from indigenous aquatic bacteria were large (>50-kb) potentially conjugative plasmids that exhibited broad-host-range capabilities (19). However, classifying such environmental plasmids primarily on the basis of transfer activity, while important for predicting the potential for horizon-

tal transfer, is not sufficient for characterizing plasmid diversity.

Molecular-based plasmid classification or replicon typing by using DNA sequences of replication origins and incompatibility loci of well-characterized plasmids originally isolated from clinical and animal environments has been shown to be useful in typing or classifying plasmids from bacterial isolates of medical importance (13, 16). Plasmids containing similar or related replication systems are considered incompatible if they cannot coexist in the same host cell (15, 36). To date, more than 30 different incompatibility groups in gram-negative bacteria, including the broad-host-range plasmid groups IncN, -P, -Q, and -W and the narrow-host-range plasmid groups IncB/O, -FIA, -FII, -FIB, -HI1, -HI2, -I1, -L/M, -X, and -U (7), have been reported. While many plasmids of medical importance have been well studied, general information is lacking on the host range, maintenance requirements, conjugal abilities, and incompatibility groupings of most plasmids present in bacteria isolated from marine and freshwater environments.

The present study was undertaken to determine the distribution of plasmids within marine microbial populations and to evaluate, by DNA hybridization, whether the incompatibility and replication (*inc*/*rep*) regions of representative broad- and narrow-host-range plasmids would be suitable for classifying plasmids isolated from culturable bacterial populations of coastal marine sediments collected from Mission Bay, San Diego, Calif. The plasmid-bearing isolates examined in this study included a wide range of gram-negative and gram-positive genera representative of marine eubacteria (*Vibrio*, *Aeromonas*, *Alteromonas*, and *Bacillus*). In addition, because the vast majority of marine bacteria are nonculturable, DNA was extracted from the microbial community and examined for the presence of broad- and narrow-host-range replicon sequences by PCR amplification.

MATERIALS AND METHODS

Marine sediment sampling and bacterial isolation. Sediment cores (10 to 15 cm in length, 5 cm in diameter) were collected at low tides from the Kendall-Frost Mission Bay Marsh Reserve site in San Diego, Calif., during June 1995, August 1995, and November 1995. The Kendall-Frost site is a coastal salt marsh under tidal influence. Urban runoff primarily affects the northern and western perimeters of the reserve. At the time of sample collection, water temperatures ranged from 21.5°C (June 1995) to 24.0°C (November 1995) and salinity was 35‰. Sediment cores were extruded into sterile Whirl-pak bags (Nasco, Fort Atkinson, Wis.), stored on ice during transport to the laboratory (<1 h), and processed immediately.

Sediment samples (1 g) were serially diluted in artificial seawater (37 g of sea salts per liter; Sigma Chemical Co., St. Louis, Mo.) (50) and spread onto the following solid media: (i) Difco (Detroit, Mich.) 2216E medium; (ii) 100-fold-diluted Difco 2216E medium (adjusted for salinity); (iii) YTSS (1% yeast extract, 0.5% tryptone) (50); (iv) half-strength YTSS (0.5% yeast extract, 0.25% tryptone); (v) TSS (0.1% tryptone); (vi) UASS (unamended seawater prepared by autoclaving 1 liter of freshly collected seawater for 30 min); (vii) YESS (0.3% yeast extract; (viii) SESS (marine sediment extract prepared by autoclaving 400 g of marine sediment for 30 min and filtering through sterile gauze); (ix) NYSS (0.8% nutrient broth, 0.5% yeast extract). With the exception of UASS and the 2216E medium, artificial seawater was used in all of the media and 1.7 to 2% agar was added when necessary. After dilutions, each sediment sample was spread onto the nine different media and the plates were incubated for 1 to 14 days at 30°C. Morphologically different colonies were picked from the plates and restreaked at least twice on the same medium to ensure purity. Typically, 30 to 60 isolates were selected from the nine different media. Sodium ion requirements were determined by the method of Baumann et al. (4). Only those isolates with sodium ion growth requirements were considered to be of marine origin. The Gram reaction of each isolate was determined as described by Powers (41). Selected bacterial isolates were identified on the basis of cellular fatty acid methyl ester content by Analytical Services (Williston, Vt.).

Isolation of plasmids from marine bacterial isolates. A modification of the method of Kieser (27) was used to screen for the presence of plasmids from the gram-negative and gram-positive marine bacterial isolates. Five to ten milliliters of an overnight cell culture, grown in the same medium in which the isolate was initially cultured, was centrifuged (6,000 × *g*, 10 min), and the cell pellet was thoroughly drained, resuspended in 500 μl of solution A (2 mg of lysozyme per ml, 0.3 M sucrose, 25 mM Tris [pH 8.0], 25 mM EDTA [pH 8.0], 0.02% bromocresol green), and incubated at 37°C for 30 min; this was followed by the addition of 250 μl of solution B (0.3 M NaOH, 2% sodium dodecyl sulfate [SDS]), mixing by inverting several times, and incubation at 55°C for 30 min. Samples were allowed to cool to room temperature prior to the addition of 180 μl of solution C (5 g of phenol, 5 ml of chloroform, 1 ml of distilled water, 5 mg of 8-hydroxyquinoline), quickly vortexed to mix, and centrifuged (8,000 × *g*, 5 min). The supernatant was carefully removed and immediately loaded onto 0.6 to 0.8% horizontal agarose gels. Gels were run at 5 V per cm, stained with ethidium bromide, destained in water, and photographed on a UV transilluminator.

TABLE 1. Broad-host-range and narrow-host-range plasmid incompatibility and replication probes used in this study

Inc group	Probe size (bp)	Plasmid source	Cross-reactivity
Broad host range			
N	1,000	R46	
P	750	RK2	
Q	357	R1162	
W	1,150	RSa	
Narrow host range			
B/O	1,600	pMU700	IncFIC, IncI1, IncK
FIA	917	F	
FII	543	R1*drd-19*	com9, IncFIC
FIB	1,202	P307	
HI1	2,250	TR6	
HI2	1,800	TP116	
I1	1,100	R64*drd-11*	IncFIC, IncB/O
L/M	800	pMU407.1	
X	942	R6K	
U	950	RA3	

Isolation of incompatibility and replication probes. *E. coli* strains containing plasmids carrying the incompatibility loci and origins of replication of a number of well-known plasmids were obtained from M. Couturier (Table 1). Plasmids were purified from 50 to 100 ml of cell culture grown in Lennox L broth (35) (Gibco Scientific, Grand Island, N.Y.) by either cesium chloride gradient centrifugation or ion-exchange columns (Qiagen-Tip 100; Qiagen, Chatsworth, Calif.) and digested with the appropriate restriction endonucleases as described by Couturier et al. (13). DNA fragments containing the *inc*/*rep* regions from 14 different plasmids were eluted from low-melting-point agarose by QIAquick gel extraction (Qiagen). The isolated DNA fragments to be used as probes were labelled by random priming with [α-^{32}P]dATP (6,000 Ci/mmol; NEN Dupont) and the Boehringer Mannheim (Indianapolis, Ind.) random-primed DNA labelling system.

Hybridizations. Following electrophoresis to screen for plasmid DNA, gels were denatured, neutralized, and blotted onto nylon membranes (Schleicher & Schuell, Inc., Keene, N.H.) essentially as described in the manufacturer's recommendations; however, DNA was routinely allowed to transfer overnight to ensure complete transfer of large (>15-kb) plasmids. To ensure transfer of large plasmids, gels were initially soaked in 0.25 M HCl for 30 min prior to denaturation. Following transfer, membranes were rinsed in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM NaCl [pH 7.7]) (33), baked for 2 h at 80°C under a vacuum, and stored until hybridization. The membranes were then washed in prewarmed (42°C) 2× SSPE solution, placed in hybridization bottles (Hybaid Instruments, Holbrook, N.Y.), and prehybridized in 30 ml of hybridization solution consisting of 50% (vol/vol) deionized formamide, 6× SSPE, 5× Denhardt's solution, 1% SDS, and 100 μg of salmon sperm DNA per ml at 37 to 42°C for 4 to 8 h at 7 rpm. Radiolabelled probes were added at approximately 2×10^6 cpm per ml and incubated at 37 to 42°C for 16 h at 7 rpm. Unbound label was removed by washing membranes twice in 2× SSPE–0.1% SDS at room temperature for 15 min and twice at 50°C in 2× SSPE–0.1% SDS for 1 h in the Hybaid oven at 10 rpm. The final wash step was in 2× SSPE at room temperature, and membranes were exposed to BioMax X-ray film (Kodak) at −70°C with an intensifying screen. When necessary to reprobe membranes with different *inc*/*rep* probes, bound labelled probe was removed as described in the manufacturer's recommendations (Schleicher & Schuell).

Screening for antibiotic and heavy metal resistance phenotypes. Antibiotic-containing disks (Difco) were applied to lawns of marine bacterial isolates on half-strength YTSS agar. The antibiotics used (and their concentrations) were as follows. The aminoglycosides were gentamicin (10 μg), kanamycin (10 μg), neomycin (30 μg), and streptomycin (10 μg). The beta-lactams were ampicillin (10 μg), penicillin (10 μg), and carbenicillin (100 μg). Other antibiotics included tetracycline (30 μg), the macrolide erythromycin (15 μg), chloramphenicol (30 μg), and trimethoprim (5 μg). Plates were scored after incubation for 18 to 24 h at 30°C, and resistance was determined by the method of Bauer et al. (3).

Resistance to the metal salts cadmium chloride, cobalt chloride, zinc chloride, nickel chloride, and mercuric chloride was assayed essentially by the rapid screening method of Summers and Jacoby (52). Briefly, a 5- by 80-mm ditch was cut in the center of the half-strength YTSS agar plate, and overnight cultures of the marine isolates were streaked from the edge of the ditch to the edge of the plate. After the streaks had dried completely, approximately 1 ml of the various metal ion solutions, with concentrations of 1, 2.5, 5, 10, 20, 25, and 40 mM for Cd^{2+}, Co^{2+}, Zn^{2+}, and Ni^{2+} and of 0.1, 0.2, 0.4, and 0.5 mM for Hg^{2+}, was added to the ditch and the plates were incubated for 24 h at 30°C. After incubation, the relative susceptibility of each isolate was determined by measuring the inhibition zones and comparing the zones of the isolates to those of the positive controls of *E. coli* SK1592 (pDU202; resistant to Hg^{2+}) and *Alcaligenes eutrophus* CH34 (pMOL28, pMOL30; resistant to Cd^{2+}, Co^{2+}, Zn^{2+}, and Ni^{2+}) (34).

DNA extraction and purification from marine sediments. Total DNA (genomic and plasmid) was extracted from sediment samples (1 to 5 g) by the method of Tsai and Olson (55) with slight modifications. Modifications to the method consisted of two phenol-chloroform extracts rather than a phenol-only and a phenol-chloroform extraction. Purification of the crude DNA extract to remove humic acids and other coextracted contaminants was done by the method of Tebbe and Vahjen (53) with ion-exchange columns (Qiagen-Tip 500).

PCR amplification. Primers (20- to 21-mers) for detection of the origin of replication for the broad-host-range plasmids RK2 (IncP) and the narrow-host-range plasmid F (IncFIC) were based on published sequences (38, 43, 51). The 5′-to-3′ sequence of IncP-1 was TGATTTTACGCGAGTTTCCC (coordinates 12517 to 12536) (38), that of IncP-2 was AAAAGACAGGTTAGCGGTGG (coordinates 12296 to 12315) (38), that of IncF-1 was TAGTATCCTCTGCGA AACGAT (coordinates 145 to 165) (43), and that of IncF-2 was GCCTGAGA TAAGCAAGAATG (coordinates 457 to 476) (43). Primers were synthesized by Operon, Inc. (Alameda, Calif.). Amplification was performed as described by Saiki et al. (44) with *Taq* polymerase (Boehringer Mannheim). The PCR solution contained 1× PCR amplification buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl [pH 8.3]), deoxynucleoside triphosphates (each at 200 μM), 1 μl of extracted sediment DNA (representing approximately 5 to 10 mg of sediment) used as DNA template, 2.5 U of *Taq* DNA polymerase per 100 μl, and T4 gene 32 protein (2.5 μg/100 μl; Boehringer Mannheim). The addition of T4 gene 32 protein which binds and stabilizes single-stranded DNA has been shown to improve PCR amplifications (53). A total of 35 PCR cycles were run under the following conditions: denaturation at 95°C for 1 min, primer annealing at 65°C for 1 min, and DNA extension at 72°C for 1 min with an initial incubation at 98°C for 5 min and 60°C for 5 min. Amplified products were detected on 1.0% agarose gels run in Tris-borate-EDTA buffer, stained with ethidium bromide, and photographed on a UV transilluminator.

RESULTS

Incidence of indigenous plasmids in marine sediment bacteria. Several studies have reported on the incidence and frequency of plasmids within marine water column and sediment bacterial isolates (2, 5, 24). However, in only a few cases has there been an attempt to classify plasmids from the marine environment by such characteristics as incompatibility group, maintenance requirements, and conjugal proficiency.

By use of a modification of the Kieser procedure (27) to isolate plasmid DNA, the frequency of plasmids in 1,086 heterotrophic marine sediment bacterial isolates cultured from coastal California salt marsh sediments ranged from 24% for November 1995 to 28% for June and August 1995 (data not shown). The method used for identifying a plasmid is based on its presence as a covalently closed circular DNA molecule. Linear plasmid DNA molecules would be less likely to be detected by the procedure used in this study. Nine different media (varying in the concentrations of organic nutrients) were used for the isolation of plasmid-bearing bacteria to obtain as much culturable bacterial diversity as possible. However, there was no difference in the frequency of plasmids observed when either low or high nutrient conditions were initially used to cultivate sediment bacterial isolates (data not shown).

Comparison of known plasmid size standards to the 297 plasmid-containing marine bacterial isolates to obtain an estimation of plasmid mass indicated a broad range of plasmid sizes (5 to >250 kb; Fig. 1A and Fig. 2). However, the majority of plasmids in the sediment bacterial isolates were generally large and ranged from 40 to 100 kb. While the frequency of

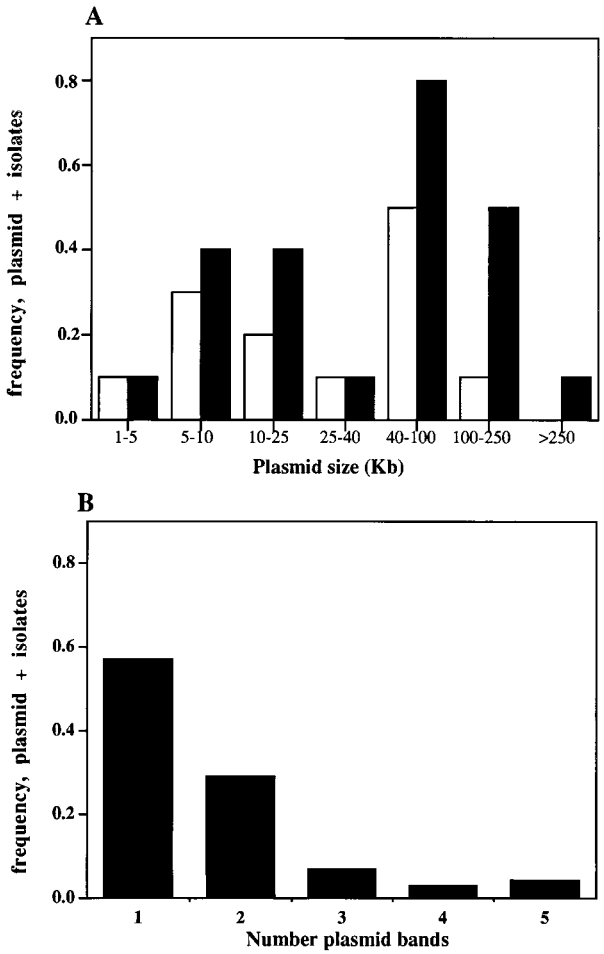

FIG. 1. (A) Plasmid size distributions for plasmid-containing marine bacteria isolated from coastal California salt marsh sediments in August 1995 (□) and November 1995 (■). (B) Incidence of single and multiple plasmids detected in plasmid-containing marine sediment bacterial isolates.

plasmids ranging in size from 5 to 40 kb did not appear to differ more than twofold between the sampling dates, there did appear to be a higher incidence (as much as fivefold for the 100- to 250-kb size range) of large plasmids (40 to >250 kb) occurring in isolates collected from the November 1995 sediments (Fig. 1A). Although the majority of isolates contained only one plasmid, numerous isolates contained multiple plasmid bands (Fig. 1B). Given the relative positions of the bands in the agarose gels, in most cases it is unlikely that they represent open circular or multimer forms of the same plasmid. Approximately 70% of the 297 plasmid-bearing isolates were gram negative. Representative plasmid-bearing isolates were identified by fatty acid analysis as belonging to the genera *Vibrio*, *Alteromonas*, *Aeromonas*, *Photobacterium*, and *Paracoccus*. In addition, some of the gram-positive plasmid-bearing isolates showed similarity in fatty acid composition to members of the genera *Bacillus* and *Streptomyces*.

Characterization of plasmids from marine isolates by incompatibility and replication probes. While a previous study (12) has demonstrated the value of replicon typing with the *inc*/*rep* probes devised by Couturier et al. (13) for plasmid-containing bacterial isolates of clinical importance, a recent study suggests some limitations of these replicon probes (14). It is uncertain whether the present incompatibility groups and

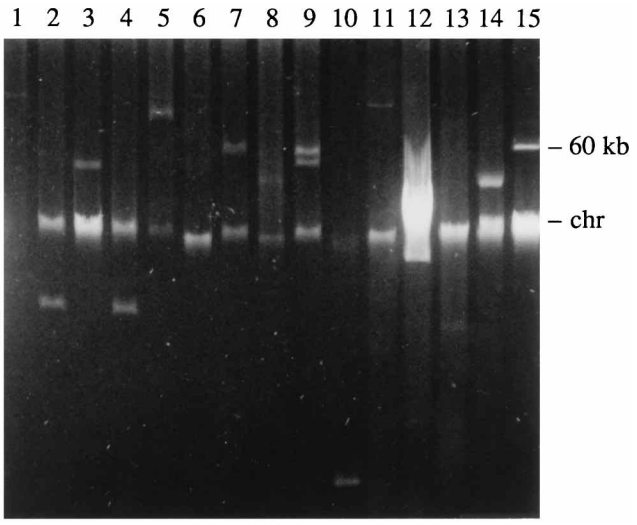

FIG. 2. Ethidium bromide-stained 0.7% agarose gel electrophoresis of representative plasmids isolated by the modified Kieser method from marine sediment bacteria (lanes 1 to 14) and *E. coli* MV10 containing the 60-kb plasmid RK2 (lane 15). The expected position of the chromosomal DNA band (chr) is indicated.

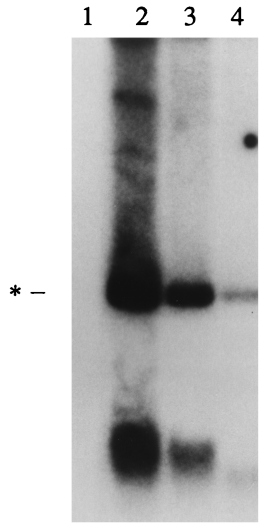

FIG. 3. Sensitivity of detection of plasmid RK2 by Southern blot analysis using the 750-bp IncP probe. Hybridization signals were obtained from plasmid DNA isolated by the modified Kieser method from 10^8 (lane 2), 10^7 (lane 3), and 10^6 (lane 4) *E. coli* MV10 cells containing RK2. No signal was detected with 10^8 plasmid-free *E. coli* MV10 cells (lane 1). The position of the supercoiled plasmid RK2 is indicated by an asterisk. The weaker bands below the asterisk are likely plasmid RK2 with either a different superhelical density or a nicked form of the plasmid.

their corresponding replicon probes are representative of plasmids within bacterial assemblages found in marine sediment ecosystems.

Following the initial isolation of plasmids from the culturable marine sediment bacterial populations, isolates were subjected to a second round of isolation to reconfirm the presence of plasmids (Fig. 2). Comparison of plasmid band intensity between the 60-kb plasmid RK2 (five to seven copies per chromosome) (54) and plasmids of similar molecular size indicated that many of the plasmids from the marine isolates are either at the same or a lower copy number relative to that of RK2 (Fig. 2). Plasmid DNA from all of the 297 plasmid-bearing isolates was subsequently subjected to Southern hybridization with each of the 14 *inc*/*rep* probes representing the origins of replication and incompatibility loci of well-studied plasmids, many of which have been derived from clinical environments (Table 1). Many of the plasmid-specific sequences used for the *inc*/*rep* probes contain regions involved in plasmid copy number (i.e., replication control) (13). Interestingly, none of the plasmids isolated from the 297 plasmid-bearing marine isolates exhibited homology to any of the 4 broad-host-range replicon probes (Table 1) or to any of the 10 narrow-host-range replicon probes (data not shown). Moreover, lowering the stringency of hybridization (i.e., <75% homology) did not reveal any indigenous marine plasmids that had significant homology to any of the *inc*/*rep* probes used in this study (data not shown).

Since it appeared that many of the plasmids isolated during this study were large (>40 kb) and present in host cells at fairly low copy numbers (e.g., <10 per chromosome), the quantity of available target sequences for hybridizations could be quite low. Therefore, by using RK2 as the large low-copy-number plasmid model, we determined the level of hybridization sensitivity in our studies. We were able to easily detect as little as 1 ng of RK2 plasmid DNA with the 750-bp *incP* replicon probe (Fig. 3 and Table 1). This amount corresponds to approximately 1 plasmid molecule in 10^6 cells (Fig. 3). The DNA loaded on the agarose gels for hybridization analysis was obtained from approximately 5×10^8 cells. Thus, it seems likely that our lack of homology between the 14 different *inc*/*rep* probes and the 297 indigenous plasmid-containing isolates was due to a lack of DNA sequence homology and not to a low plasmid copy number limiting the target sequence concentration.

While several studies have confirmed the utility of the various *inc*/*rep* probes for classifying plasmids from bacterial isolates of clinical and animal origins, we are unaware of any reports attempting to use *inc*/*rep* plasmid sequences to type plasmids isolated from marine sediment bacterial populations. Kobayashi and Bailey (28) recently reported that only one-third of plasmid-bearing bacterial isolates collected from the sugar beet phyllosphere had homology to one or more of the *inc*/*rep* probes representing the narrow-host-range repFIIA and repFIB incompatibility groups. Our observed lack of hybridization between the various *inc*/*rep* probes and the 297 culturable plasmid-bearing marine isolates strongly suggests that plasmids isolated from the salt marsh ecosystem contain quite different replication and incompatibility genes. Thus, the present set of plasmid probes based predominantly on incompatibility groups derived from members of the family *Enterobacteriaceae* is probably not representative of the type of plasmids occurring in marine sediment microbial communities.

Plasmid replication sequences in microbial community DNA. Typically, we were able to culture approximately 0.01% of the total bacterial population (based on acridine orange direct counts; data not shown) regardless of either the initial isolation medium used or the particular sampling date. This result is not surprising since the vast majority of marine bacteria are not readily cultured (18, 26). Although we were unable to detect plasmids that were homologous to currently available incompatibility plasmid groupings in the culturable bacterial fraction, we attempted to determine by PCR amplification whether broad- and narrow-host-range plasmid replication and incompatibility sequences were present in the DNA extracted from the microbial community of coastal marine sediments.

Community DNA was screened by PCR amplification for two different plasmid replication and incompatibility regions. The broad-host-range 60-kb plasmid RK2 was selected since it is known to be highly promiscuous in self-transfer and can replicate and be stably maintained in a diverse background of gram-negative bacteria (46). The second plasmid employed was the 100-kb narrow-host-range plasmid F, which is able to replicate only in *E. coli* and closely related bacteria (22). The F plasmid was selected due to its prevalence (as much as 50% in all isolates) in bacteria cultured from fecal and sewage samples (32). To determine the detection limit of target sequences amplified from plasmid replication regions with the PCR conditions used in this study, we inoculated sterilized sediment samples with either F- or RK2-containing *E. coli* cells at levels ranging from 0 to 1.0×10^8 cells per g of sediment. PCR products from approximately 1,000 cells containing RK2 were visible on agarose gels (Fig. 4A, lane 4). The sensitivity of detection was determined to be from 10 to 100 cells per g (wet weight) by Southern blot analysis of amplification products (data not shown). Similar results were obtained for sterilized sediment samples seeded with *E. coli* cells containing the F plasmid (Fig. 4A, lanes 9 to 11).

While we were unable to detect a positive signal from microbial community DNA extracted from the sediment samples obtained in August and November 1995 and probed with F replication-specific primers (Fig. 4A, lanes 12 and 13), we could detect a positive signal with RK2 replication-specific primers (Fig. 4A, lanes 5 and 6). The nature of the PCR product obtained with the RK2 primers was confirmed by Southern hybridization with a 167-bp RK2 *oriV* sequence probe (data not shown). To examine sequence variability within the amplified RK2 *oriV* region, the 240-bp PCR product was digested with the restriction enzyme *Sau*3AI, which, if the restriction site was present, would yield a 102-bp fragment and a 138-bp fragment (Fig. 4B). The PCR product that we were able to amplify from the DNA extracted from the salt marsh sediment microbial community was cleaved by *Sau*3A and yielded fragments of the expected size, suggesting that the diversity of the RK2 *oriV* region in the sediment microbial community is either low or not readily detected by the method that we employed. Deviation in the sequence of RK2 *oriV* is not expected since studies on RK2 *oriV* activity have indicated that the sequence requirements are relatively stringent (39). Because we routinely isolate plasmid DNA such as RK2 from laboratory-grown bacterial strains, it is imperative to control for PCR false positives caused by laboratory cross-contamination of community DNA extracted from the marine sediments. To detect any such contamination possibility, we included autoclave-sterilized sediment samples as negative controls during each sediment extraction assay to monitor for contamination of RK2 plasmid sequences. We did not detect any RK2 contamination of the sterilized sediment samples during our studies (Fig. 4A, lanes 7 and 14).

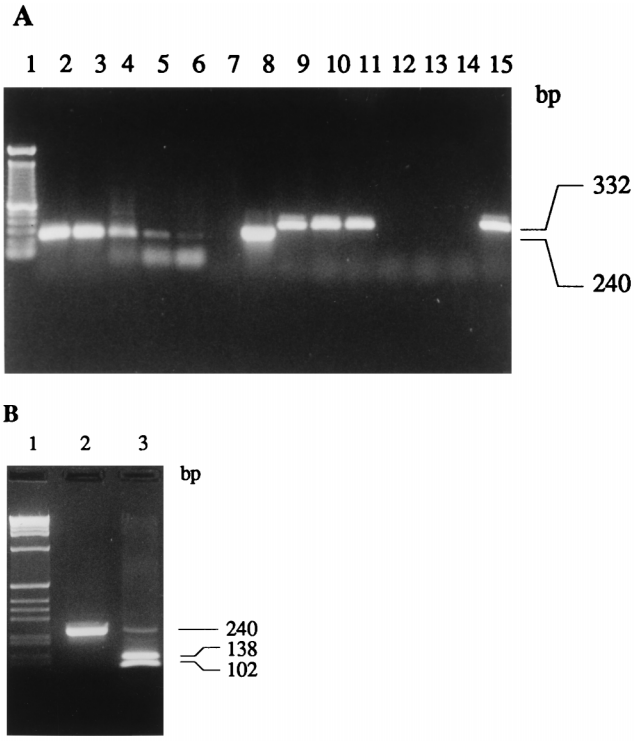

FIG. 4. Amplification of plasmid replication sequences from DNA extracted from marine sediment microbial communities. (A) Ethidium bromide-stained 1% agarose gel electrophoresis of PCR-amplified DNA using either RK2-specific primers (lanes 2 to 8) or F-specific primers (lanes 9 to 15). Lanes (numbers in parentheses indicate estimated amount of seeded cells amplified after dilution of the extract for the PCR): 1, 100-bp DNA size standard; 2, seeded with 10^8 *E. coli* cells containing RK2 per g (10^6); 3, seeded with 10^7 cells per g (10^5); 4, seeded with 10^5 cells per g (10^3); 5, total community DNA extracted from 1 g of sediment collected in August 1995; 6, total community DNA extracted from 1 g of sediment collected in November 1995; 7, negative control (sterilized sediment; no template DNA added); 8, positive control (cesium chloride-purified RK2 plasmid DNA); 9, seeded with 10^8 *E. coli* cells containing F plasmid per g (10^6); 10, seeded with 10^7 cells per g (10^5); 11, seeded with 10^5 cells per g (10^3); 12, total community DNA extracted from 1 g of sediment collected in August 1995; 13, total community DNA extracted from 1 g of sediment collected in November 1995; 14, negative control (sterilized sediment; no template DNA added); 15, positive control (cesium chloride-purified F plasmid DNA). (B) Restriction enzyme digestion (*Sau*3AI) of amplified RK2 *oriV* region from total microbial community DNA. Lanes: 1, 1-kb DNA size standard; 2, RK2 *oriV* PCR product; 3, *Sau*3AI-digested RK2 *oriV*-amplified product from total microbial community DNA.

Phenotypic characterization of indigenous plasmids. The occurrence of antibiotic- and metal-resistant bacteria in coastal marine sediments and waters has often been used as a pollution indicator (2, 17). However, while the resistance is often associated with plasmids, curing and reintroduction of plasmids to conclusively prove plasmid-encoded resistance traits generally have not been carried out. All of the 297 plasmid-bearing marine sediment isolates were analyzed for antibiotic resistance as well as heavy metal tolerance. Resistance to tetracycline, the aminoglycosides kanamycin and streptomycin, and to the beta-lactams penicillin, ampicillin, and carbenicillin was found most frequently (Table 2). Resistance to neomycin, erythromycin, trimethoprim, and chloramphenicol was detected less frequently. However, when 150 putative plasmid-free isolates were analyzed to determine if there was a general correlation between plasmid content and antibiotic resistance phenotypes, similar frequencies of drug resistance were observed (Table 2). While the percentages of plasmid-containing isolates exhibiting multiple antibiotic resistances ranged from a high of 52% for penicillin, ampicillin, kanamycin, and tetracycline to a low of 1% for penicillin, ampicillin, and gentamicin, similar percentages were obtained for the plasmid-free bacterial isolates (data not shown). While it is possible that the alkaline lysis procedure used in this study to identify plasmids from the marine bacterial isolates may not have detected plasmids considerably greater in size than 250 kb, there does not appear to be any obvious correlation between plasmid content and antibiotic resistance. A similar lack of correlation between plasmid content and heavy metal resistance was also observed in the plasmid-containing and plasmid-free marine sediment bacterial isolates (Table 3), with the possible exception of

TABLE 2. Percentage of antibiotic-resistant plasmid-containing and plasmid-free marine sediment bacterial isolates

Antibiotic	% of isolates resistant to antibiotic	
	Plasmid containing (n = 297)	Plasmid free (n = 150)
Ampicillin	52.0	39.8
Carbenicillin	48.0	31.5
Chloramphenicol	1.2	3.7
Erythromycin	28.0	14.5
Gentamicin	34.0	35.4
Kanamycin	52.0	53.9
Neomycin	19.3	25.8
Penicillin	48.0	38.7
Streptomycin	43.0	50.6
Tetracycline	47.0	38.8
Trimethoprim	24.0	25.9

mercuric chloride resistance. Plasmid-bearing isolates exhibited a fivefold increase (11.6%) in mercuric chloride resistance relative to that of plasmid-free sediment isolates (2.0%) (Table 3).

DISCUSSION

Previous studies have demonstrated the presence of plasmids in marine sediment and water column bacterial populations. Numerous studies have also attempted to correlate phenotypic characteristics, including antibiotic, heavy metal, and UV resistances, to plasmids of environmental origins (2, 29, 40, 47). It appears from these studies that plasmids present in marine sediment and water column bacterial populations generally do not carry these readily assayed selective traits; instead, their function(s) remains largely unknown. In this study, we have undertaken to genotypically characterize plasmids from marine sediment bacterial populations by molecular analysis of their incompatibility and replication properties. Replicon typing with 14 broad- and narrow-host-range *inc*/*rep* plasmid groups was employed to determine if plasmids of marine origin contain *inc*/*rep* regions homologous to those of previously characterized replicon groups (7, 13, 15, 16). Information pertaining to the basic biology of environmental plasmids could facilitate the use of these natural replicons in areas such as bioremediation, the design of genetically engineered microbes, and large-scale industrial processes. Approximately 30% of more than 1,000 marine bacterial isolates cultured from coastal sediments contained one or more plasmids. Plasmids were detected in such marine genera as *Vibrio*, *Aeromonas*, *Alteromonas*, and *Bacillus*. While Kobori et al. (29) previously reported as much as a twofold increase in the frequency of plasmid incidence in Antarctic bacterial isolates initially cultivated on lower nutrient conditions, we did not detect any difference in the plasmid frequencies of isolates initially isolated under either the low- or high-nutrient conditions used in our study. Moreover, we were unable to detect any strong correlation between plasmid content and such phenotypic traits as antibiotic and heavy metal resistance in the 297 plasmid-containing marine sediment bacterial isolates.

TABLE 3. Percentage of heavy metal-resistant plasmid-containing and plasmid-free marine sediment bacterial isolates

Metal	% of isolates resistant to metal	
	Plasmid containing (n = 297)	Plasmid free (n = 100)
Cadmium	40.7	29.1
Cobalt	19.8	33.3
Mercury	11.6	2.0
Nickel	43.0	43.8
Zinc	55.8	39.6

Interestingly, our hybridization findings have indicated a lack of homology between the replication and maintenance genes of plasmids occurring in culturable marine sediment bacterial populations and well-characterized replicons of clinical importance. Chaslus-Dancla et al. (12) reported a good correlation between probe homology and standard incompatibility mating assays to classify plasmids from isolates of clinical or animal origin with the *inc*/*rep* probes. Recent findings by Amuthan and Mahadevan (1) reported that 77% of plasmids isolated from phytopathogenic *Xanthomonas* spp. could be molecularly typed with the *inc*/*rep* probes. Kobayashi and Bailey (28) reported that 31% of plasmid-containing bacterial isolates collected from the sugar beet phyllosphere and identified as *Klebsiella* and *Erwinia* spp. hybridized to the narrow-host-range *inc*/*rep* replicon probe repFIB or repFIIA.

Our findings indicate that plasmids isolated from the microbial communities in coastal marine sediments encode novel replication and incompatibility loci that lack homology to clinically derived plasmid incompatibility groups. It is conceivable that plasmids of marine sediment origins encode replication and maintenance genes that are either unique or even more diverse than those of their previously characterized clinical counterparts. Assuming that the metabolic burden of maintaining large plasmids requires that plasmids confer some selective advantage to the host bacterium, it seems likely that plasmids maintained in the marine sediment bacterial populations are encoding diverse and advantageous traits to the host cell that may not be readily identified. In addition to encoding partitioning mechanisms to ensure stable plasmid maintenance, a number of studies have described postsegregational cell killing systems in broad-host-range and several narrow-host-range plasmids that promote self-maintenance by prohibiting the formation of plasmid-free daughter cells that may arise due to replication and partitioning errors (9, 21, 30, 42). It is of considerable interest to determine if plasmids in marine sediment bacteria use unique or similar mechanisms to provide for their stable maintenance.

Several studies have detected either IncP1 plasmids or IncP1-related DNA sequences in isolates cultured from both terrestrial and aquatic ecosystems (10, 25, 45). While we were unable to detect RK2 sequences within the 24 to 28% of culturable bacterial isolates containing plasmids that were homologous to the IncP probe, we were able to amplify, from total environmental DNA, RK2-like *oriV* sequences from the marine sediment microbial community DNA. Further analysis of the amplified PCR product indicated that the sequence was similar to the well-characterized plasmid RK2 originally isolated from clinical environments (38). Recently, Gotz et al. (21a) have also been able to amplify, by PCR, IncP plasmid-specific sequences from some soil and manure slurries. It is possible that the RK2 replication sequences amplified from the microbial community DNA is occurring in the form of free DNA (adsorbed to sediment particles and protected from nuclease digestion) (31). In addition to the possibility that RK2-like plasmids are present in the nonculturable bacterial fraction of the marine sediment microbial community, it is also possible that RK2 DNA is present as a wash-in from sewage contamination of the coastal salt marsh environment. However, this latter possibility would seem less likely since the IncF sequences were not detectable in the same samples. Regard-

less of the source of the RK2 replication sequences, our findings would suggest that broad-host-range replicons such as RK2 are not found at a high frequency within the marine sediment bacterial population.

The lack of hybridization observed between more than 300 plasmids isolated from a salt marsh marine sediment bacterial community and the classical incompatibility and replication regions of well-characterized plasmids of clinical and animal origins indicates that the present classification system is not adequate for typing plasmids of coastal marine (sediment) origins. We are presently characterizing incompatibility and replication origins of the marine sediment plasmids obtained from this study which may be used as replicon probes for future studies. By applying replicon probes from plasmids of environmental origins, we will be better able to determine the extent of plasmid diversity in marine sediment ecosystems.

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