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Conjugative Plasmids Isolated from Bacteria in Marine Environments Show Various Degrees of Homology to Each Other and Are Not Closely Related to Well-Characterized Plasmids

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Mercury resistance plasmids were exogenously isolated, i.e., recovered after transfer to a model recipient bacterium, from marine air-water interface, bulk water, and biofilm communities during incubation in artificial seawater without added nutrients. Ninety-five plasmids from different environments were classified by restriction endonuclease digestion, and 12 different structural plasmid groups were revealed. The plasmid types isolated from different habitats and from different sampling occasions showed little similarity to each other based on their restriction endonuclease patterns, indicating high variation and possibly a low transfer between microhabitats and/or a different composition of the microbial communities at different sites and times. With another approach in which probes derived from one of the isolated plasmids and a mercury resistance (*mer*) probe from Tn*501* were used, similarities between plasmids from several different groups were found. The plasmids were further tested for their incompatibility by use of the collection of *inc*/*rep* probes (B/O, com9, FI, FII, HI1, HI2, I1, L/M, N, P, Q, U, W, Y) described by Couturier et al. (M. F. Couturier, P. Bex, L. Bergquist, and W. K. Maas, Microbiol. Rev. 52:375–395, 1988). Hybridizations did not reveal any identity between the 12 plasmid groups and any of the *inc*/*rep* probes tested. The results indicate that plasmids isolated from different marine habitats have replication and/or incompatibility systems that are different from the well-characterized plasmids that are commonly used in plasmid biology. This shows the need for the use of more relevant plasmids in studies of plasmid activity in the environment and development of new *inc*/*rep* probes for their characterization.

The abundance of plasmids in bacteria from diverse environments, as well as the potential role that plasmids have in the adaptation of bacteria during changing environmental conditions, is well established. Horizontal transfer of genes has a major impact on the adaptability of bacteria, exemplified by the dissemination of antibiotic resistance genes. Other compounds exert different selective pressures in the environment, and plasmids carrying genes for functions such as heavy metal resistances and catabolic pathways are often found (34, 35). Knowledge about plasmid distribution, evolutionary relationships, and diversity of plasmids in relation to the natural selection pressures is needed to understand the role of plasmids in the flow of genetic information in natural bacterial communities. Studies in pursuit of such knowledge require that plasmids can be identified and classified. This has been done for plasmids present in bacteria of medical importance and has revealed groups of plasmids that have similarities in housekeeping functions. One approach to such a characterization is based on plasmid incompatibility. This is an inherited property of all plasmids (8, 25), and the classification is facilitated by DNA hybridization of plasmids with a series of incompatibility (Inc) group-specific probes (5).

Attempts have been made to classify plasmids isolated from soil and wastewater by use of these group-specific probes (13, 19, 31, 32) as well as plasmids from marine salt marsh sediments (30). Recently, PCR primers for some of the incompatibility groups have been designed and used in detection of broad-host-range plasmids from manure and soil samples (12). It has been stressed that studies on incompatibility of plasmids isolated from different habitats are needed before the full extent of the diversity of natural plasmid replicons can be assessed (19).

The aim of this work was to investigate the distribution and variation of conjugative plasmids from different marine bacterial communities. Plasmids conferring mercury resistance were isolated from bulk water, biofilms, and the air-water interface by the exogenous isolation method (10). The marine interface microenvironments are relevant because interfaces in general have been suggested to be sites where gene transfer would be frequent due to high cell densities (16). In addition, the air-water interface is shown to accumulate mercury in high concentrations (15, 16, 21). Characterization of the plasmids was performed to detect similarities, differences, and variation between different environments and times. Methods such as restriction endonuclease digestion for grouping of the plasmids and different probes to assess similarities were used. We especially wanted to test whether the plasmids corresponded to common incompatibility groups because this would greatly assist their classification.

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MATERIALS AND METHODS

Exogenous isolation of conjugative plasmids carrying mercury resistance. *Pseudomonas putida* UWC1 (22) was used as model recipient bacterium. UWC1

is a derivative of *P. putida* KT2440 and is a plasmid-free, restriction-negative, and rifampin-resistant strain (1).

UWC1 was grown in Luria broth with extra salt (LB20; yeast extract, 5 g; tryptone, 10 g; NaCl, 20 g [each per liter of deionized water]). Solidified media contained 1.5% (wt/vol) agar with the appropriate antibiotic. Artificial seawater, termed NSS (NaCl, 17.6 g; Na_2SO_4; 1.47 g; $NaHCO_3$, 0.08 g; KCl, 0.25 g; KBr, 0.04 g; $MgCl \cdot 6H_2O$, 1.87 g; $CaCl_2 \cdot 2H_2O$, 0.41 g; $SrCl \cdot 6H_2O$, 0.01 g; H_3BO_3, 0.01 g [each per liter of deionized water]) (24), was used for washing, starvation, dilutions, and resuspension of bacteria from filters. Where appropriate, sterile mercury chloride, 25 μg/ml (Hg^{2+}), and/or rifampin, 100 μg/ml, was added to the media.

Bacterial community samples were collected from the shoreline or from a boat close to the shore near Göteborg, Sweden. Samples 1, 2, and 3 were collected in August (bulk water), September (bulk water and air-water interface), and November (bulk water and biofilm) 1993, respectively. The salinity was about 20‰, and the area is affected by the outflow of the river Göta Älv. The mercury concentration in the river water was below 0.1 μg per liter at the time of sampling, but enrichment of Hg in a *Fontanalis* sp. indicated a strong influence of Hg on higher organisms in the area (Göta Älvs vattenvårdsförbund). Air-water interface samples were collected by lowering a sterile glass plate vertically into the water and then slowly withdrawing it vertically from the surface, and the attached water film was wiped off with a neoprene wiper (14). The thickness of the water film corresponds to a sampling depth of about 50 μm. Bulk water samples were collected by opening a bottle about 30 cm below the surface. Biofilm samples were taken by scrubbing stones, collected at about a 50-cm depth, as described previously (4).

Viable cells were determined as CFU on selective plates incubated at 25 to 28°C. The number of recipients, donors, and transconjugants were determined after 3 days of incubation. Total cell numbers were determined by the standard acridine orange direct count method (AODC).

Model recipient bacteria (UWC1) were grown overnight, with shaking, in LB20 with rifampin at 25°C. The cells were then washed twice by centrifugation and then resuspended in equal volumes of NSS, giving about 10^9 cells per ml, incubated in NSS, with shaking, overnight at room temperature, and mixed with the marine donor bacteria the following day.

The biofilm, air-water interface, and bulk samples were prefiltered through 25- and 10-μm polyester filters (Monodur; AB DERMA) to remove larger particles and organisms. The cell densities of the natural samples and the model recipient were determined by AODC; the samples were then adjusted to give the same total numbers of donors in the paired air-water interface–bulk and biofilm-bulk sample incubations. The numbers of recipients were also adjusted to be the same for each paired-sample incubation, such that ratios of transconjugants per donor cell could be compared. The mixture of recipients and donors was deposited onto a polycarbonate filter (0.2-μm pore size; Poretics Corp.) and floated on 20 ml of NSS in a sterile petri dish (11).

After 48 h of incubation at room temperature, the cells on the filters were resuspended by vortexing in 2 to 5 ml of NSS and serial dilutions were plated on the appropriate plates. Transconjugants were isolated on Hg-rifampin plates, potential donors were isolated on Hg, and recipients plus transconjugants were isolated on rifampin. The plates were incubated at room temperature. Controls were made by checking spontaneous mutation frequencies of UWC1 by plating on selective media. Randomly picked colonies of UWC1 were identified with the API 20 NE system (bio Mérieux sa, Marcy l'Etoile, France) to verify their origin. Plate mating, i.e., plasmid transfer on the selective plates, was tested by incubating recipients and donor bacteria directly on selective plates.

Restriction endonuclease characterization of plasmids. Ninety-five randomly selected transconjugants from the different habitats and sampling occasions were analyzed for plasmid content with the Qiagen midiprep kit (Diagene). Plasmid preparations were run on 0.4% agarose Tris-borate-EDTA (TBE) gels and then stained with ethidium bromide. Plasmids from the same preparations were then digested with *Eco*RI and *Sal*I, in accordance with the manufacturer's instructions. The digested DNA was separated on ethidium bromide-stained 0.8% agarose TBE gels. The plasmids were grouped by their restriction patterns.

Transconjugants with plasmids representing each restriction group were further analyzed on Eckhardt gels (9) together with a supercoiled DNA ladder (GIBCO BRL) and the plasmids RP4 and pDK9 (source, Kaye Wachsmuth) as references for size determinations of the plasmids and to test the presence of additional, very large plasmids.

Determination of transfer abilities. One representative plasmid from each structural group was tested for transfer abilities. Mating experiments of the plasmids from *P. putida* UWC1 to the recipient strain *P. putida* KT2440 (Nx^r; ATCC 47054) were performed on polycarbonate filters. Donors and recipients from overnight cultures were mixed at ratios of 1:10, and a total volume of 1 ml was deposited onto filters and incubated on LB20 plates for 2 h at room temperature. The filters were vortexed in 1.5% NaCl, and bacteria were enumerated on appropriately amended LB20 plates. In addition, one of the plasmids that had a relatively small size was also purified from an agarose gel fragment and transformed into *P. putida* KT2440 by electroporation (17) and tested for transfer ability to *P. putida* UWC1.

Probes, Southern blotting, and hybridization. *inc*/*rep* DNA probes (B/O, com9, FI, FII, HI1, HI2, I1, L/M, N, P, Q, U, W, Y) described by Couturier et al. (5) were used for replicon typing of the plasmids. Probe DNA was prepared as described previously (5) and labelled by digoxigenin random-primed labelling (Boehringer Mannheim).

Probes for the mercury resistance *mer* operon (from a 2.3-kb *Eco*RI fragment from Tn*501*; pBR322::Tn*501*) were labelled by the same method.

Probes for the *tnpA* genes from Tn*21* and Tn*501* are described elsewhere (6). Two probes were also prepared from one of the characterized plasmids (group 1, see below).

Plasmid DNa was digested with *Sal*I, fragments were separated by agarose gel electrophoresis, and fragments of <10 and >10 kb were labelled separately.

Southern blots were obtained from 0.8% agarose TBE gels of *Eco*RI- or *Sal*I-digested plasmid DNA. The gels were subjected to vacuum blotting (Pharmacia) onto negatively charged nylon membranes (Boehringer Mannheim). Control DNA from the probe plasmid collection was fixed on membranes by dot blotting. Agarose gels of the plasmids, RP4 (IncP) digested with *Kpn*I and *Sac*II, pSa (IncW) digested with *Bam*HI and *Sac*II, and R46 (IncN) digested with *Bgl*II, were also subjected to Southern blotting.

Hybridization was performed with the respective probe under standard conditions as described in the manufacturer's instructions (Boehringer Mannheim). Hybridization was detected by chemiluminescence (Boehringer Mannheim) using X-ray film (Fuji).

TABLE 1. Gene transfer frequencies of Hg^{2+} resistance from natural bacterial communities to model recipients

Sample no.	Origin of donors (community)	No. of recipients/ filter	Total no. of donors (by AODC)/ filter	Frequency[a]
1	Bulk water	4.4×10^9	5.0×10^9	2.3×10^{-8}
2	Bulk water	1.0×10^{10}	4.0×10^9	5.0×10^{-10}
	Air-water interface	1.7×10^{10}	4.0×10^9	7.0×10^{-9}
3	Bulk water	1.9×10^{11}	7.0×10^9	2.1×10^{-8}
	Biofilm	2.3×10^{11}	7.0×10^9	2.6×10^{-8}

[a] Number of transconjugants per total number of donors added initially (measured by AODC).

RESULTS AND DISCUSSION

Exogenous isolation of Hg^r plasmids. All of the marine communities showed transfer of Hg^r plasmids to the model recipients (Table 1). The transfer occurred in artificial seawater, without added nutrients and to starved recipients. Nongrowing conditions during the matings mimic the nutrient-limiting conditions prevailing in marine waters (23). The isolated plasmids are ecologically relevant in that they are likely to be active under natural conditions and are relevant for further characterization. Rich laboratory media are often used in exogenous isolation, but this may lower the diversity of the isolated plasmids because a fraction of the donor community or the recipients may outgrow potential donors and also because the growth of transconjugants results in clones with identical plasmids. Transfer frequencies at the different occasions and from the different sites were in the range of 5×10^{-10} to 2×10^{-8} transconjugants per total number of donors (Table 1). The frequency of spontaneous mutations was below 10^{-11} for UWC1 on mercury. Randomly picked transconjugants from different samples were tested by use of the API 20 NE system and confirmed the reisolation of UWC1. No transconjugants were detected when plate mating was tested, showing that gene transfer occurred during incubation in artificial seawater and not on the selective plates. The survival of UWC1 throughout the gene transfer experiment was about 50%.

Structural grouping. Ninety-five randomly selected *P. putida* UWC1 transconjugants from the different habitats and samplings (Table 2) were tested for plasmid content. All of the tested transconjugants showed plasmid bands on the agarose gels. The isolated plasmids were all <100 kb in size, and the majority were around 60 kb (see Table 4). No additional, very

TABLE 2. Distribution of plasmids in different structural groups, and among sample occasions and sampling sites, defined by restriction analysis patterns

Plasmid group[a]	No. of plasmids isolated from[b]:					Total no. of plasmids/ structural group
	Sample 1 from bulk	Sample 2		Sample 3		
		Bulk	A/W	Bulk	BF[f]	
1[c]	50			6		56
2[c]			13			13
3			4			4
4		1		2		3
5					2	2
6					2	2
7					2	2
8			1		1	2
9	1					1
10				1		1
11				1		1
12					1	1
13 (ND)[d]	1	1			5	7
Total no. of plasmids tested	52	2	18	10	13	95

[a] Groups are defined by distinctly different patterns after digestion with *Eco*RI and *Sal*I.

[b] The numbers of plasmids isolated from samples 1, 2, and 3 for the sampling sites bulk water community (Bulk), air-water interface community (A/W), and biofilm community (BF) were determined. The total numbers of plasmids isolated were 112, 2, 28, 140, and 180 for sample 1, sample 2—bulk, sample 2—A/W, sample 3—bulk, and sample 3—BF, respectively.

[c] One plasmid lacks one band from the general structural pattern after endonuclease digestion.

[d] ND, not determined due to few or no fragments generated by endonuclease digests.

large plasmids were found when representatives from each structural group were run on Eckhardt gels (data not shown) (9). The 95 plasmids could be divided into 12 structural groups by their restriction endonuclease digest patterns (Table 2 and Fig. 1A). The different groups were defined such that their digest patterns were distinctly different when *Eco*RI and *Sal*I were used (Fig. 1A shows the restriction pattern for *Sal*I). Two plasmids had digest patterns that differed from their general group pattern by one band; these two plasmids were still placed in their respective general groups. Some plasmid digestions generated no or only one fragment with both enzymes. These plasmids could therefore not be classified and were placed in group 13 (Table 2). The sizes of the plasmids coincided with their structural grouping in that the plasmids from the same group were of the same size. In cases in which plasmids within a group had a missing band compared to the pattern of the general group, those plasmids were also smaller than the usual plasmids in that group. Plasmid grouping based on the restriction endonucleases *Eco*RI and *Sal*I gave a good resolution of the plasmids. However, this method gave a minimum number of plasmid groups since the use of additional enzymes might have revealed more differences.

Mercury resistance plasmids have been exogenously isolated from other environments such as river epilithon (summarized in reference 10) and from sugar beets (20). These plasmids were also characterized by restriction endonuclease digests, and structural groups were identified. However, the majority of the plasmids examined in these studies were from 100 to more than 300 kb in size, while the plasmids examined in the present study were all smaller than 100 kb.

Table 2 shows the distribution of different plasmid groups within the sampled habitats. The transconjugants from bulk samples 1 and 2 had the lowest numbers of plasmid types; for the 52 transconjugants in sample 1, only two plasmid groups were clearly distinguished. The 13 biofilm transconjugants represent five different plasmid groups. The plasmids isolated from the biofilm sample belong to different groups than plasmids from the bulk sample taken at the same time. The same was also true for the air-water interface and bulk transconjugants in sample 2. This might be due to the fact that bacteria in these two habitats do not rapidly exchange plasmids or that the composition of these communities differ. It is interesting that new groups of plasmids are found in every sampling (Table 2). Only three different plasmid types could be found in more than one sample or habitat. The use of one strain as the recipient may reduce the potential number of isolated plasmids because all plasmids may not be stable in this strain. This possibility, together with the fact that only one marker was used, indicates a rather large variation and transfer potential of plasmids among different microenvironments. The differences in plasmid groups found on different sampling occasions might also reflect a seasonal variation in the composition of the bacterial community. Such variations in marine waters have been recorded earlier with 16S rRNA probes (28).

Transfer abilities. Representatives from all 12 different groups showed transfer to *P. putida* KT2440 at frequencies ranging from 2.5×10^{-4} to 2.2×10^{-6} after 2 h of mating (Table 3). It was shown that the plasmid in group 12 is, in fact, conjugative despite its small size, 24 kb, and a similar frequency of transfer was observed with *P. putida* KT2440 after transformation into this strain compared with transfer from the original transconjugant.

Replicon typing. Hybridizations of the 12 representatives from the grouping of the 95 Hg^r plasmids with the probes for the 14 different *inc*/*rep* groups gave no positive signals. The *inc*/*rep* probes were originally derived from bacteria isolated from clinical and animal environments. The probes have been used in other investigations of environmentally isolated plasmids. For example, of 79 plasmid-containing strains from sugar beet phyllosphere, 25 hybridized to 3 of 14 tested probes, but 54 isolates showed no signal with the tested replicon probes (19). Replicon typing of conjugative plasmids with mobilizing abilities, isolated from soil and activated sludge, showed that only one of four plasmids from polluted soils but all three tested plasmids from activated sludge hybridized to the IncP probe (31). Also, five of six 2,4-dichlorophenoxyacetic acid-degradative plasmids isolated from soil belonged to IncP (32). Two hundred ninety-seven plasmids isolated from bacterial isolates from marine salt marsh sediments on the California coast showed no homology with the *inc*/*rep* probes (30). Investigations of plasmids isolated from soil (19, 31), from marine sediments (30), and now also from bulk water, air-water interface, and biofilms show that the currently available replicon probes are not sufficient to classify these plasmids.

Presence of *mer* and *tnpA* genes on the plasmids. Representatives from 10 of the 12 distinct plasmid groups hybridized with the *mer* probe from Tn*501* (Table 4 and Fig. 1B). The *mer* operon from Tn*501* was chosen because it was shown to be involved in the adaptation of aquatic bacteria to mercury (2) and because Tn*501* and Tn*21* *tnpA* sequences are found in seawater (6). The presence of this *mer* operon on different plasmids may be a result of transposition of Hg^r-containing transposons from one plasmid to another. One plasmid from group 11 did hybridize to the *tnpA* probe mixture (Table 4). We have earlier shown that Tn*501* and Tn*21* *tnpA* sequences were present in total DNA both from bulk seawater and from the air-water interface (6), and a recent investigation showed a widespread distribution of Tn*501*- and Tn*21*-related *tnpA* and

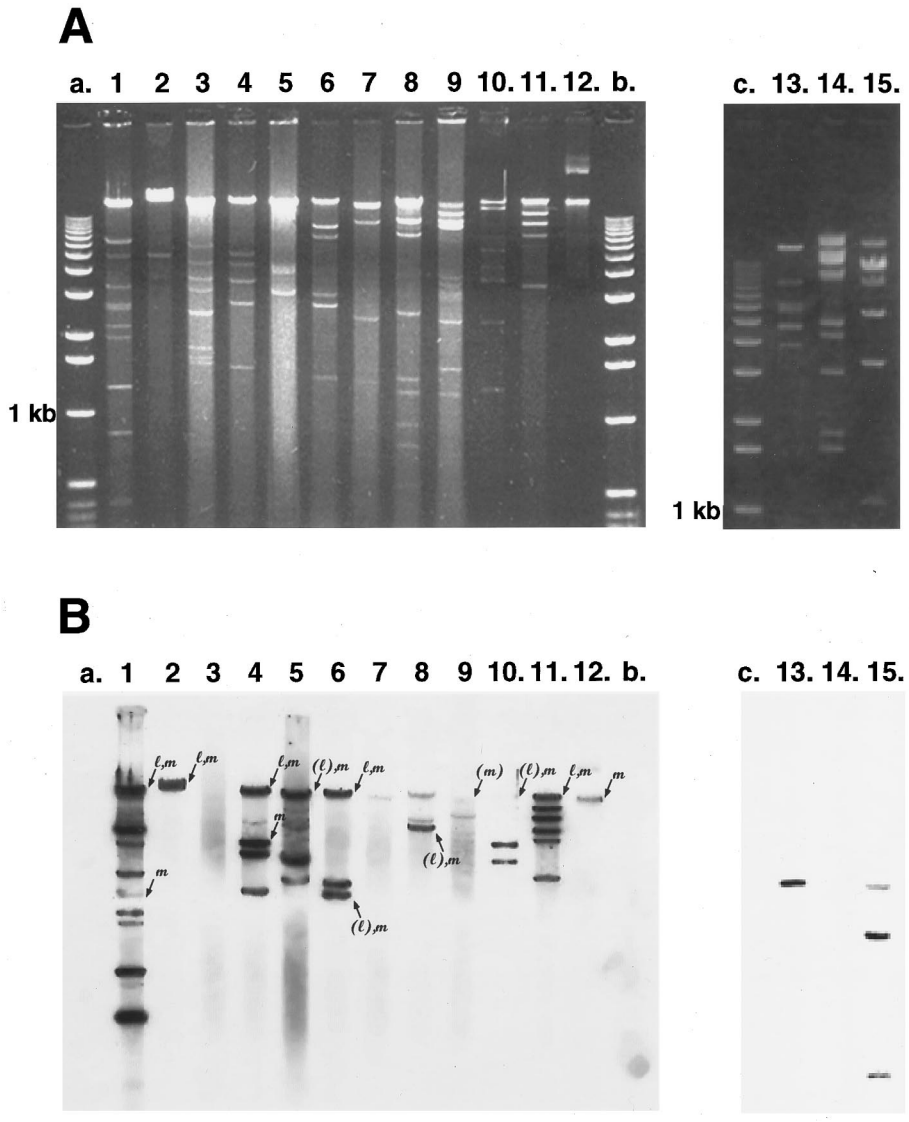

FIG. 1. Illustration of agarose gels of *Sal*I-digested plasmids from structural groups 1 to 12 and of digestions of three control plasmids and corresponding Southern hybridizations with probes derived from a group 1 plasmid. (A) Ethidium bromide-stained gels of *Sal*I-digested plasmids. Lanes: 1 to 12, structural groups 1 to 12, respectively; 13 to 15, digests of plasmids R46 (*Bgl*II), RP4 (*Kpn*I and *Sac*II), and pSa (*Bam*HI and *Sac*II), respectively; a to c, molecular size marker 1 kb. (B) Autoradiograph with labelled group 1 plasmid DNA (fragment sizes, <10 kb) following the hybridization of the digests of structural groups 1 to 12 (lanes 1 to 12, respectively) shown in panel A. Bands hybridizing to the large group 1 plasmid probe (>10 kb) and the *mer* probe are indicated with l and *m*, respectively; these letters in parentheses indicate weak hybridization signals. In lanes 9 and 10, the arrows indicate the positions of the bands hybridizing with the large group 1 plasmid probe and the *mer* probe but not the <10-kb probe. An autoradiograph with labelled group 1 plasmid DNA (both <10 and >10 kb in size) is also shown (lanes 13 to 15). The band visible in lane 13 contains the genes for resistance to spectinomycin, ampicillin, and sulfonamide of plasmid R46. In lane 15, the visible bands from the plasmid pSa digest contain part of the sulfonamide resistance gene, the chloramphenicol resistance gene, and a small gene involved in conjugation (top band), genes for resistance to sulfonamide (partial), specinomycin, and kanamycin (middle band), and genes of unknown function (lower band).

tnpR sequences in mercury-resistant bacterial isolates from soil (27). It has earlier been shown that *mer* sequences hybridizing to probes derived from Tn*501* are highly variable (26). The hybridizations with the *mer* probe (Fig. 1B) indicate this because fragments of different sizes hybridize with the probe in different plasmid groups. This implies that the information gained by the *mer* probe hybridizations presented here do not necessarily mean that the *mer* genes present on the plasmid are of recent evolutionary relation. Due to the spreading of mercury resistance, possibly via transposons, to many different plasmids, the mercury resistance phenotype is useful when plasmid diversity is investigated.

Hybridization with probes derived from a group 1 plasmid. Probes for determining similarity between the 12 structural groups were derived from a plasmid from group 1. This structural group was chosen due to its high number of representatives in the plasmid collection. This plasmid has also been shown to transfer to and be maintained in *Escherichia coli* (unpublished results) in addition to *Pseudomonas*, which makes it broad host range by definition (7, 18).

Hybridizations with the two probes from a group 1 plasmid revealed various degrees of homology between the plasmid groups. The presence of *mer*-like determinants on the group 1 plasmid is probably responsible for some of these positive

TABLE 3. Transfer frequencies of representative plasmids from each structural group after 2 h of mating

Structural group no.	No. of transconjugants/donor[a]
1	1.6×10^{-5}
2	2.3×10^{-4}
3	5.5×10^{-5}
4	2.4×10^{-4}
5	1.3×10^{-5}
6	2.5×10^{-4}
7	2.2×10^{-6}
8	5.5×10^{-5}
9	1.5×10^{-4}
10	1.4×10^{-4}
11	3.2×10^{-5}
12	1.8×10^{-4}

[a] Transfer frequencies are the means of two independent experiments and are calculated as the number of transconjugants recovered divided by the number of donor bacteria present when mating was initiated.

hybridizations. Five of the plasmid groups (groups 2, 4, 5, 6, and 11) had high degrees of homology to the probes. The hybridization of, for example, group 11 showed an extensive homology to group 1 plasmid, and the number of large fragments hybridizing to the probes is too high to be accounted for only by the *mer* determinants. This high similarity between group 1 and group 11 was not evident in comparisons of the restriction endonuclease digest patterns (Fig. 1). In the cases of four plasmid groups (groups 8, 9, 10, and 12), a lower degree of homology was observed, some of which probably is due to the *mer*-like determinants (Fig. 1B). The two remaining plasmid groups (groups 3 and 7) did not show any positive hybridization to either the group 1 plasmid probes or to the *mer* probe. The lack of clear homology between some of the plasmid groups and the group 1 probes implies that at least two different unknown incompatability groups are represented in the plasmid collection. It is, however, not possible to say whether the positive hybridizations are due to plasmid backbone genes such as replication or transfer determinants or to associated genes as, for example, resistance determinants.

TABLE 4. Plasmid size and presence of *mer* and *tnpA* genes on representative plasmids from each structural group

Plasmid group	Plasmid size (kb)[a]	Presence of[b]	
		mer[c]	*tnpA*[d]
1	93	+	−
2	51	+	−
3	86	−	−
4	51	+	−
5	59	+	−
6	61	+	−
7	59	−	−
8	59	+	−
9	39	(+)[e]	−
10	66	+	−
11	72	+	+
12	24	+	−

[a] Determined by agarose gel electrophoresis.
[b] Genes were present (+) or absent (−) on plasmids based on hybridization signals.
[c] Determined by positive hybridization signal with a *mer* probe from the Tn*501mer* operon (2.3-kb *Eco*RI fragment from Tn*501*; pBR322::Tn*501*).
[d] Determined by positive hybridization signal with *tnpA* probe from Tn*501* and Tn*21* (6).
[e] (+), weak hybridization signal.

The control plasmids RP4 (29), pSa (33), and R46 (3) were digested so that plasmid replication and transfer genes were separated from the resistance determinants. The hybridization of plasmid pSa with the two group 1 probes produced a strong signal with the fragment containing the resistance genes for spectinomycin, kanamycin, and a part of the sulfonamide resistance gene (Fig. 1). A weaker signal was obtained with the larger fragment containing the second part of the sulfonamide resistance gene, the gene for chloramphenicol, and a small gene involved in conjugation (Fig. 1). A positive signal was also obtained with a small fragment with unknown function (Fig. 1). In the case of the plasmid R46, a single fragment containing the resistance genes for spectinomycin, ampicillin, and a part of a sulfonamide resistance gene hybridized with the probes (Fig. 1). Plasmid RP4 did not give rise to any positive hybridization signals. The genes coding for the major parts of transfer functions did not hybridize to the probes derived from the group 1 plasmid for any of the three control plasmids. This shows that the transfer system of this plasmid, as well as the *inc*/*rep* sequences, are different from those of the well-characterized broad-host-range plasmids of the IncP, -W, and -N groups.

Conclusions. The plasmids studied here showed a considerable variation based on their restriction endonuclease digest patterns. Plasmids from closely located microhabitats had little resemblance to each other, based on restriction digests. The DNA-DNA homology between a plasmid from one group and those from the other groups, on the other hand, revealed that high degrees of homologies to some of the other plasmid groups could be found. The restriction endonuclease digest patterns of these plasmids had little resemblance to the pattern of the probe plasmid. This shows that precaution should be taken when plasmid diversity is assessed by restriction fragment length polymorphism.

This study also shows that the plasmids isolated from marine environments cannot be characterized by the presently available replicon probes. Additional probes need to be defined such that replicon typing can be applied. Incompatibility and replication determinants, rather than host beneficial genes, must still be considered the best specific targets for plasmid classification because they reveal the core of the plasmid housekeeping genes.

To obtain better knowledge about plasmid biology in natural habitats, it is necessary to investigate plasmids that are relevant for these specific environments. This is, to our knowledge, the first report in which plasmids have been exogenously isolated during conditions that mimic the natural environment, i.e., in artificial seawater without added nutrients. This will ensure that the plasmids are actively transferred under natural conditions and not only in high-nutrient laboratory media. These plasmids will be used in further experimental work to investigate their transfer during ecologically relevant conditions.

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