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Frequency and Characteristics of Plasmids in Bacteria Isolated from Deep-Sea Amphipods

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Bacterial strains isolated from deep-sea amphipods were identified, classified, and screened for plasmid content. Plasmids were common, with 11 of 16 isolates carrying one or more plasmids; these ranged in size from 2.9 to 63 megadaltons. Several of the strains demonstrated distinctly different phenotypic traits yet contained plasmids of the same molecular weight. Results of agarose gel electrophoresis, DNA hybridization, and restriction analysis indicate that the plasmids detected in these deep-sea isolates are identical, suggesting that transmission may occur in the deep-sea environment and that plasmids are common in some deep-sea habitats.

Plasmid transfer between bacteria provides a mechanism by which genetic information can be transmitted from one organism to another. Conjugation and the movement of DNA from donor to recipient are complex processes which have been extensively studied and thoroughly reviewed (6, 28–30). Previous studies have demonstrated the movement of resistance plasmids through bacterial populations present in or associated with health care and farming facilities, where selective pressure arises from widespread use of antibiotics for therapeutic and/or prophylactic purposes (8, 24).

Whereas plasmid transmission between bacteria of different genera has been demonstrated in the laboratory (2, 9, 11, 20, 22–24), it is not considered to be a common occurrence in the environment, except in the case of R-plasmid transfer among bacteria during sewage treatment processes (1, 4, 15). Extensive reports of the incidence of plasmids in deep-sea bacteria have not been published. Results of this study suggest that bacteria isolated from the gut of abyssal amphipods frequently harbor plasmids and that interspecies transmission of plasmids may occur in situ.

Bacterial isolates. Samples were collected during the BIOGASS 11 cruise sponsored by CNEXO, Brest, France, at depths of 4,300 m at station 3 in the Bay of Biscay Abyssal Plain (J. Deming, submitted for publication; G. T. Rowe and S. W. Deming, J. Marine Res., in press) (Fig. 1). Bacterial strains were isolated from the gut contents of amphipods of the genus *Eurythenes* captured in a baited, free vehicle trap retrieved from 4,300 m. Three of the crustaceans were aseptically dissected, and their digestive tracts, with contents, were pooled and macerated in 2 ml of Marine Broth 2216 (Difco Laboratories) enriched with dibutylphthalate (10 μg/liter), bis(tributyltin) oxide (1 μg/liter), or quinone (1 μg/liter). After incubation at 1 atm (101.29 kPa) and 3°C, purification and isolation of individual colonies were achieved with UBYE medium (19). Colonies appearing to be morphologically distinct were selected for further study, and stock cultures were prepared and stored in liquid nitrogen.

Media. Strains subjected to plasmid analysis were grown in a broth consisting of 1% tryptone, 0.5% yeast extract, 1% NaCl, 0.4% MgCl, and 0.1% KCl (LBV). This medium was also used to prepare plates after the addition of 1.3% agar (LBVA).

Plasmid detection. Plasmid carriage was examined by the gentle lysis procedure of Clewell and Helinski (7), the rapid lysis procedures of Kado and Liu (13) and Birnboim and Doly (3), and an alkaline denaturation procedure developed in our laboratory. The supernatant was aspirated, and the pellet was suspended in a 100-μl solution containing 2 mg of lysozyme per ml, 0.01 M EDTA, 0.025 Tris hydrochloride, and 0.05 M glucose. The mixture was incubated on ice for 15 min, after which lysis was achieved by gently layering 200 μl of the following solution onto the cell suspension: 0.01 M Tris hydrochloride–0.04 M EDTA–4% sodium dodecyl sulfate (pH adjusted to 12.9 with 1.0 N NaOH). Mixing was accomplished by rolling the tube between the palms of the hands until the suspension cleared. The lysate cleared in a few seconds and was neutralized with 52 μl of 2 M Tris hydrochloride (pH 7). The tube was again rolled gently until a reduction in viscosity was noted. The sodium dodecyl sulfate-protein-DNA complex was precipitated with 100 μl of 5 M NaCl and incubated on ice for 1 h. The precipitate was pelleted in a microfuge for 3 min; the tubes were rotated 180° within the head and centrifuged 3 additional min to produce a compact pellet. The supernatant was transferred to a clean tube, and an equal volume of fresh phenol prepared by the method of Maniatis et al. (17) was added. The contents were mixed gently by rolling the tubes, not by vortexing or emulsification. Separation was achieved by centrifugation for 5 min, and the upper phase was collected and transferred to a clean tube. After the addition of 0.1 volume of 3 M potassium acetate and 2.5 volumes of ethanol, the tubes were chilled at −70°C for 30 min.

After centrifugation, DNA pellets were suspended in 25 to 35 μl of Tris-EDTA buffer containing 30 μg of heat-treated RNase per ml and incubated at 37°C for 15 min. Tracking dye (50% glycerol, 1% sodium dodecyl sulfate, 0.1 M EDTA, 0.25% bromophenol blue, xylene cyanol, and orange G) was added, and the samples were heated at 65°C for 5 min. Samples were run in 0.4 to 0.8% agarose gels in acetate buffer (17).

For restriction enzyme digestions, the DNA pellets were suspended in appropriate buffer; enzyme was added, and the preparation was incubated at 37°C for 1 h. RNase solution (10 μl) was added, and the mixture was incubated for 10 min at 37°C. The reactions were stopped by the addition of ⅙

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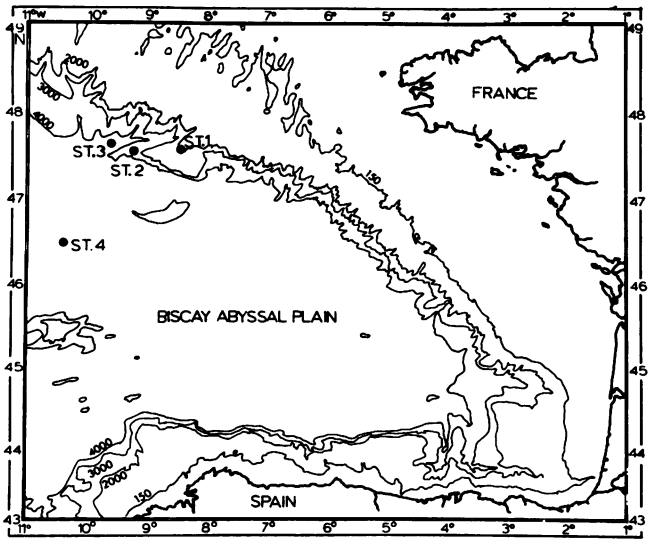

FIG. 1. Stations sampled during the BIOGASS 11 cruise.

volume of tracking dye, followed by heating to 65°C for 5 min.

Isolation of plasmid DNA for nick translation. Large-scale plasmid extraction was performed, and the resulting lysates were placed in a preparative agarose gel (0.6%) and run at 3 V/cm until adequate separation was achieved. Plasmid DNA was visualized by placing the ethidium bromide-treated gel on a UV transilluminator. The gel containing the plasmid band was carefully removed and placed in a dialysis bag. The DNA was electroeluted by the method of Schlief and Wensink (21). The contents of the bag and three subsequent washes with TE buffer were passed twice through an Elutip column (Schleicher & Schuell Co., Keene, N.H.). The plasmid DNA was recovered from the column according to the manufacturer's instructions. DNA was nick translated with [δ-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.). Enzymes and unlabeled deoxyribonucleotides were obtained from Bethesda Research Laboratories, Gaithersburg, Md. Transfer of electrophoretically separated DNA to Zeta-probe membranes (Bio-Rad Laboratories, Richmond, Calif.) and subsequent hybridizations were performed by the method of Southern (25). Hybridizations were performed at 45°C for 18 h; stringent washes were carried out at 15°C below the melting point (73.8°C). Autoradiograms were obtained with Kodak XAR-5 film and a DuPont Cronex light intensifying screen; the film was exposed for 24 h at −70°C.

Taxonomic studies. Identification and classification of the isolates were done as suggested by West and Colwell (27). Chromosome extractions and mole-percent G+C determinations were performed on isolates D6-2 and D42-2 by the method of Marmur and Doty (18), with a Guilford model 2600 spectrophotometer (Guilford Instruments, Oberlin, Ohio). The results were 58.6 and 50% G+C, respectively.

All of the bacteria isolated from the amphipod gut were gram-negative, oxidase-positive rods and, with the exception of three strains which were not motile, possessed a polar flagellum. Based on results of the taxonomic tests, it was possible to divide the isolates into two major groups (Table 1). The first group was comprised of pseudomonas-like isolates, which were resistant to vibriostatic agent O/129, did not ferment glucose, and did not require NaCl for growth or demonstrate a lysine decarboxylase but did possess arginine decarboxylase. The second group, vibrio-like isolates, were inhibited by O/129, fermented glucose to acid but not gas, required NaCl for growth, possessed lysine decarboxylase, and lacked arginine decarboxylase.

The isolates from the amphipod digestive tracts were screened for the presence of plasmids by the gentle lysis procedure (7), the method of Kado and Liu (13), the method of Birnboim and Doly (3), and an alkaline lysis procedure developed during this study. Plasmid molecular weights

TABLE 1. Phenotypic traits of bacterial strains isolated from amphipod digestive tracts

Group[a] and strain no.	Gram reaction	Cytochrome oxidase	Polar flagellum	Resistance to O/129 concn (mM) of:			Growth at 42°C	Growth in % NaCl of:				Acid from glucose	Amylase	Gelatinase	Lysine decarboxylase	Acid from mannose	Arginine decarboxylase
				10	50	100		0	6	8	10						
PLI																	
D3	−	+	+	+	+	+	−	+	+	+	−	−	−	+	−	−	+
D6-1	−	+	+	+	+	+	−	+	+	−	−	−	−	+	−	−	+
D6-2	−	+	+	+	+	+	−	+	+	−	−	−	−	+	−	−	+
D9-1	−	+	−	+	+	+	−	+	+	+	+	−	−	+	−	−	+
D9-2	−	+	+	+	+	+	−	+	+	−	−	−	−	+	−	−	+
D21-1	−	+	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−
VLI																	
D21-2	−	+	+	−	−	−	+	−	+	+	+	ND[b]	ND	−	+	ND	−
D24-1	−	+	+	+	+	+	−	−	+	−	−	+	ND	−	+	+	−
D24-2	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
VLI																	
D27-1	−	+	+	+	−	−	+	−	+	−	−	+	ND	−	+	ND	−
D39	−	+	−	−	−	−	−	−	+	−	−	+	+	−	+	+	−
D42	−	+	+	−	−	−	−	−	+	−	−	+	+	−	+	−	−
D42-2	−	+	+	−	−	−	−	−	+	−	−	+	+	−	+	−	−
D45-1	−	+	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−
D45-2	−	+	+	−	−	−	−	−	+	−	−	+	−	−	−	−	+

[a] PLI, Pseudomonas-like isolates; VLI, vibrio-like isolates.
[b] ND, Not determined.

were determined by averaging measurements obtained from three different gel preparations, each of which contained plasmid standards of known molecular weight, e.g., *Escherichia coli* V517 (16) and *E. coli* J53(R386).

The plasmid contents of the isolates are given in Table 2. Of the 16 isolates, 11 contained one or more plasmids with half of the plasmid molecular sizes 28 megadaltons (MDa) or larger. Plasmids with the same molecular weight were repeatedly isolated from the 16 strains examined in this study. Five vibrio-like isolates contained various combinations of 28-, 43-, and 63-MDa plasmids. Three pseudomonas-like isolates also carried extrachromosomal DNA of 63 MDa.

The frequency of occurrence of plasmids in bacteria isolated from amphipod digestive tracts was high, and the total amount of plasmid DNA in these strains was sizeable, since the organisms contained either a single large plasmid or several small plasmids. The frequency of plasmid occurrence was greater than that reported for isolates from samples collected in unpolluted estuarine and marine waters (10, 12, 14).

Preparations to which the four different extraction procedures were applied did not always demonstrate all of the plasmid bands that could be detected in given strains by any one of the methods. The rapid isolation procedures yielded more reliable results. Plasmids of 63 MDa isolated from strains D6-1, D6-2, D9-1, and D42-2 were unstable, when stored after isolation, even in the presence of 0.01 M EDTA; they completely degraded within 3 days. DNA isolated by the gentle lysis procedure was relatively more labile.

Kobori et al. (14), who examined Antarctic isolates for plasmids, showed that the nutrient composition of the primary isolation medium can affect retention of plasmids by plasmid-carrying bacteria, with low-nutrient media yielding a greater percentage of isolates with extrachromosomal DNA. In this study, enrichment with organic chemicals was employed with deep-sea isolates. It is possible that genes encoding degradation of these chemicals may be carried on plasmids, but the plasmids isolated in this study must be considered to be cryptic until further work is done to answer this question.

Plasmid DNA was difficult to isolate from the amphipod isolates with the gentle lysing procedure of Clewell and Helinski (7), and chromosomal or plasmid DNA was rarely observed in the CsCl gradients prepared from D6-1, D6-2, or D9-1 lysates and never observed in preparations from D42-2 when this method was used. When DNA was isolated by the previously outlined procedure from D6-1, D6-2, and D9-1, it degraded within 3 days in the presence of 0.01 M EDTA and in the absence of any detectable bacterial contamination. Isolation of plasmids was improved by the addition of 0.04 M EDTA to the lysis buffer, elimination of incubations at elevated temperature after lysis, and inclusion of a phenol extraction. The results suggest that deep-sea bacteria may contain potent DNases which interfere with plasmid isolation, a finding which is being pursued further in our laboratory.

TABLE 2. Plasmids detected in bacterial strains isolated from amphipod gut samples

Strain no.	Plasmid size(s) (MDa)[a]
D3	PND[b]
D6-1	63
D6-2	63
D9-1	2.9, 5, 11
D9-2	63
D21-1	2.9, 3.3, 4.6, 5.2, 6.5, 8.3, 12.4
D21-2	28, 63
D24-1	28, 43
D24-2	PND
D27-1	43, 63
D27-2	PND
D39	28, 43
D42	PND[c]
D42-2	63
D45-1	3.7, 8.3
D45-2	PND

[a] Average values obtained from three measurements.
[b] PND, Plasmids not detected.
[c] A 63-MDa plasmid was detected only in one of the three separate isolations.

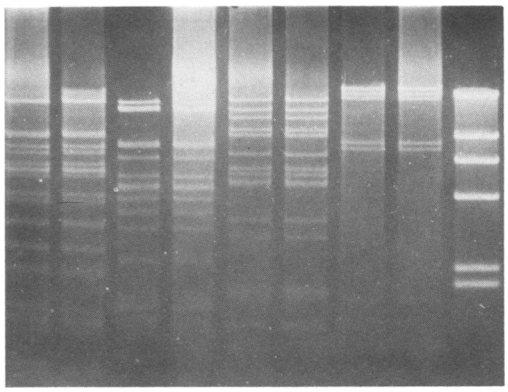

FIG. 2. Agarose gel electrophoresis of restriction digests prepared from lysates of strains D6-2 and D42-2 in which only a 63-MDa plasmid could be detected. The pairs of bands, from left to right, represent digests of restriction enzymes *Hin*dIII, *Pst*I, *Ava*I, and *Bam*HI, respectively. The rightmost column is a λ *Hin*dIII digest.

Further analyses of the large plasmids of strains D6-2 and D42-2 were done. D6-1 and D9-1 were not studied further because phenotypic traits of these strains suggested that they were closely related or identical to D6-2. Overall DNA G+C contents as determined for isolates D6-2 and D42-2 by the method of Marmur and Doty (18) were 58.6 and 50% mol, respectively.

When rapid lysis procedures were used to examine the plasmid content of these two strains, results often varied. For example, preparations of single-colony isolates of D42-2 frequently did not yield the 63-MDa plasmid. Colonies of D6-2 and D42-2, which demonstrated the 63-MDa plasmid by the rapid lysis procedure, were streaked onto three plates of LBVA and incubated for 2 days at 22°C. Twenty individual colonies of each strain were picked from the plates and extracted to determine plasmid content. The 63-MDa plasmid was detected in 19 of 20 colonies from D6-2, whereas only 3 of 20 D42-2 colonies yielded the plasmid.

The rapid lysis procedure was used to produce plasmid preparations which were digested with restriction enzymes *Hin*dIII, *Pst*I, *Ava*I, and *Bam*HI (Fig. 2). Digests with a given enzyme gave identical results.

A gel containing plasmids isolated from D6-2, D21-1, *E. coli* V517, D39, D42-1, and D45-1 (Fig. 3) was blotted onto a Zeta-probe nylon membrane (Bio-Rad) by the method of Southern (25). Plasmid DNA was isolated from D6-2 by agarose gel purification and column chromatography (21, 26). The plasmid was nick translated with [α-^{32}P]ATP (Amersham) with the protocols and reagents recommended by Bethesda Research Laboratories. Hybridizations were

performed at 45°C for 18 h; two 6-h washes were performed at 74.8°C by the method of Southern (25). The resulting autoradiogram showed two bands corresponding to the position of the 63-MDa plasmid in D6-2 and D42-2 (Fig. 4). Both bands appeared approximately equal in intensity, suggesting significant homology.

The molecular weights of plasmid DNA observed in isolates D42-2 and D6-2 were identical. DNA-DNA hybridization demonstrated that the plasmids shared extensive regions of homology. Furthermore, restriction digestion with four different six-base cutters revealed an identical band pattern for each of the enzymes. These results led to the conclusion that the same plasmid was present in strains D6-2, a pseudomonas-like isolate, and D42-2, a vibrio-like isolate. This plasmid may be common to bacteria of deep-sea amphipods, or plasmid transfer might occur in the deep sea.

Very large areas of the world oceans comprise a nutrient-poor environment (5). Nutrients entering the deep sea are far less concentrated than in estuarine or near-shore areas of the ocean. Since little or no primary production occurs in the abyssal depths, sinking detrital material from the euphotic zone is considered to comprise the predominant nutrient source. During descent into the abyss, detrital materials are degraded, with the residue being primarily recalcitrant material with relatively low nutritional value. Degradation of such recalcitrant substances by gut flora of deep-sea animals might be facilitated if the gut bacteria carry metabolic plasmids. Gene exchange among strains of the gut flora of deep-sea inhabitants, such as the amphipods, would promote a stable bacterial community by allowing members to draw on a larger genetic pool, providing concerted response to intermittent occurrence of such nutrients.

In an estuarine environment, Glassman and McNichol (10) found a greater percentage of bacteria carrying large plasmids when disturbed; i.e., polluted areas were compared with stable, unpolluted sites. The plasmids detected in bacteria isolated from polluted sites were often large enough to encode functions required for self-transmission, implying that the bacterial response to a disturbed environment may include genetic exchange to acquire needed metabolic capabilities. Because many plasmids carry insertion sequences and these sequences are also widely distributed among bacterial chromosomes, conjugative plasmids can effect the movement of large pieces of DNA. Such activities would aid in the dissemination of less common but beneficial genetic determinants and would be of significant value to the bacteria and their hosts in unstable or extreme environments.

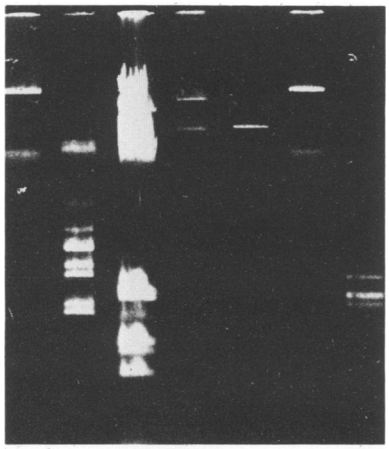

FIG. 3. Agarose gel electrophoresis of lysates from selected deep-sea bacterial strains. The lanes, from left to right, represent D6-2, D21-1, *E. coli* V517, D39, D42-1, D42-2, and D45-1, respectively.

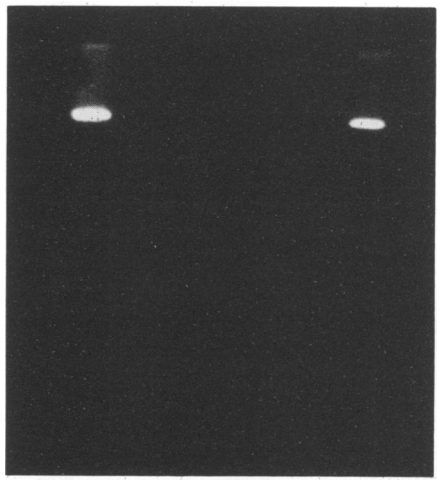

FIG. 4. Autoradiogram of Southern blot prepared from the agarose gel shown in Fig. 2.

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