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Maintenance of Plasmids pBR322 and pUC8 in Nonculturable *Escherichia coli* in the Marine Environment

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Maintenance of plasmids pBR322 and pUC8 in *Escherichia coli* that was nonculturable after exposure to seawater was studied. *E. coli* JM83 and JM101, which contained plasmids pBR322 and pUC8, respectively, were placed in sterile artificial seawater for 21 days. Culturability was determined by plating on both nonselective and selective agar, and plasmid maintenance was monitored by direct isolation of plasmid nucleic acid from bacteria collected on Sterivex filters. *E. coli* JM83 became nonculturable after incubation for 6 days in seawater yet maintained plasmid pBR322 for the entire period of the study, i.e., 21 days. *E. coli* JM101 was nonculturable after incubation in seawater for 21 days and also maintained plasmid pUC8 throughout the duration of the microcosm experiment. Direct counts of bacterial cells did not change significantly during exposure to seawater, even though plate counts yielded no viable (i.e., platable) cells. We concluded that *E. coli* cells are capable of maintaining high-copy-number plasmids, even when no longer culturable, after exposure to the estuarine or marine environment.

Extensive studies of the survival and maintenance of plasmid DNA in *Escherichia coli* have been done with both batch and continuous culture (3, 4, 9). The information which has been gathered is useful for industrial microbiology applications but does not elucidate the ecology of plasmids in the environment. Furthermore, from the work published, it is not clear whether plasmids are maintained during starvation or dormancy of microorganisms in the natural environment.

Recently, papers showing that plasmids are stably maintained in *E. coli* incubated in well water (2) and river water (7) and in *Klebsiella* spp. introduced into agricultural drainage water (22) have been published. In some cases, all plasmids were maintained, even after incubation for 203 days in a microcosm (2). Selection against survival of strains carrying plasmids, compared with that against plasmidless strains, was not observed (6). Methods used to detect plasmids in general require culturing the host cells, which may not always be possible, especially after long-term exposure in the natural environment. Although stability of plasmids has been demonstrated in stressed cells of *E. coli* (2, 7), Hill and Carlisle (10) have shown that when enrichments are employed, plasmid loss may occur.

E. coli has been demonstrated to enter a viable but nonculturable state under certain environmental conditions (5, 23). High salinity appears to be one factor that triggers *E. coli* into this state (23). Recent emphasis on planned introductions of genetically engineered microorganisms into the environment for bioremediation and other applications makes it imperative that the questions of whether microorganisms survive and whether their plasmids persist under environmental conditions be answered. In this study, the ability of nonculturable cells to retain their plasmids was investigated by employing environmental conditions simulating the marine aquatic environment.

MATERIALS AND METHODS

Inoculation of microcosms. *E. coli* JM83(pBR322) and *E. coli* JM101(pUC8) were grown in 0.5% yeast extract containing 100 μg of ampicillin per ml for 18 h at 37°C. Cells were harvested by centrifugation and washed three times in filtered (0.2-μm pore size), autoclaved 15‰ (15-g/liter) Instant Ocean (Aquarium Systems, Mentor, Ohio). Filtered (0.2-μm pore size), autoclaved 35‰ Instant Ocean (1.5 liters) was added to a 2-liter screw-cap flask. Microcosms were inoculated with approximately 1.5×10^6 cells per ml of washed culture. All microcosms were incubated in shaken culture (100 rpm) at 15°C.

Bacterial counts. Bacteria were enumerated directly by using the acridine orange direct count (AODC) method of Hobbie et al. (11). The presence of culturable bacteria was determined by plate counts on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.) with or without 100 μg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml. When the number of culturable cells obtained by plate counts dropped below 10^2 CFU/ml, culturable counts were obtained by the three-tube most-probable-number method (1), with a medium composed of 0.5% yeast extract. All plates and tubes were incubated at 35°C for 2 days by following standard bacteriological methods, after which they were examined for growth (1). No growth was detected upon extended incubation in tubes which were negative at 2 days.

Plasmid isolation by alkaline lysis. A 100-ml volume of the microcosm was filtered through Sterivex-GS (0.22-μm-pore-size) filters (Millipore Corp., Bedford, Mass.) at fixed time intervals. Ten milliliters of SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris hydrochloride [pH 7.6]) (18) was passed through the filter, after which the filters were capped at both ends, as described by Somerville et al. (20). All filters were stored at −20°C until plasmid extraction was performed. The following were added to each filter: 1.5 ml of SET buffer, 28 μl of 1 N NaOH, 40 μl of sodium dodecyl sulfate solution (20% in double-distilled H_2O), and 6 μl of DNase-free RNase (10 mg/ml). The filters were incubated on a roller (20) at 4°C for 30 min. Crude lysates were withdrawn, and the filters were washed with an additional 1 ml of SET buffer. Lysate and wash were pooled in a 15-ml Corex tube, and 1.5 ml of 3 M sodium acetate was added. The tubes were gently mixed by inversion and retained on ice for 30 min, after which the tubes were centrifuged at 17,000 × *g* at 4°C for 15 min. The

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TABLE 1. Survival of *E. coli* JM83(pBR322) and stability of pBR322 in 35‰ Instant Ocean at 15°C[a]

Incubation time (days)	Direct count	Culturable count (CFU/ml)	% Ampicillin resistant[b]
0	2.9×10^6	9.8×10^6	100
2	3.1×10^6	1.8×10^5	89
4	3.0×10^6	1.0×10^2	50
6	1.9×10^6	2.4×10^0	ND
9	2.1×10^6	$<3.2 \times 10^{-2}$[c]	ND
11	1.6×10^6	$<3.2 \times 10^{-2}$	ND
14	1.8×10^6	$<3.2 \times 10^{-2}$	ND
17	2.2×10^6	$<3.2 \times 10^{-2}$	ND
21	1.2×10^6	$<3.2 \times 10^{-2}$	ND

[a] All cultures tested positive for the presence of plasmid, as determined by agarose gel electrophoresis.
[b] (CFU per milliliter on TSA plates containing 100 μg of ampicillin per ml/CFU per milliliter on unamended TSA plates) × 100. ND, Not determined.
[c] Limit of detection was no tubes showing growth by the three-tube most-probable-number method.

TABLE 2. Survival of *E. coli* JM101(pUC8) and stability of pUC8 in 35‰ Instant Ocean at 15°C[a]

Incubation time (days)	Direct count	Culturable count (CFU/ml)	% Ampicillin resistant[b]
0	3.6×10^6	6.3×10^6	100
2	1.6×10^6	3.2×10^6	100
4	1.9×10^6	9.1×10^5	83
6	1.4×10^6	1.6×10^5	87
9	1.2×10^6	4.0×10^3	65
11	1.9×10^6	1.3×10^2	ND
14	1.8×10^6	4.3×10^{-1}	ND
17	2.1×10^6	4.0×10^{-2}	ND
21	2.0×10^6	$<3.2 \times 10^{-2}$[c]	ND

[a] All cultures tested positive for the presence of plasmid, as determined by agarose gel electrophoresis.
[b] (CFU per milliliter on TSA plates containing 100 μg of ampicillin per ml/CFU per milliliter on unamended TSA plates) × 100. ND, Not determined.
[c] Limit of detection was no tubes showing growth by the three-tube most-probable-number method.

supernatants were placed in clean tubes, and an equal volume of isopropyl alcohol was added. The tubes were gently mixed by inversion and incubated at −70°C for 30 min. Plasmid DNA was collected by centrifugation at 20,000 × *g* at 4°C for 15 min. Supernatant was discarded, and the pellets were washed successively with 5 ml each of 70, 95, and 100% ethanol. Each pellet was dried and suspended in 200 μl of sterile double-distilled H_2O. Samples were dried to 50 μl and stored at −20°C until analyzed.

Agarose gel electrophoresis. Plasmids were detected by agarose gel electrophoresis. For all samples, the concentration of agarose used was 1.0% SeaKem HTG agarose (FMC Corp., Philadelphia, Pa.). All gels were run in 1× Tris-acetate-EDTA buffer (16).

RESULTS

Results of experiments carried out to establish whether *E. coli* could survive and maintain plasmids in artificial seawater (35‰) showed that *E. coli* JM83(pBR322) entered the nonculturable state within 6 days after introduction into artificial seawater (Table 1). The limit of detection was defined as the point at which less than 0.03 culturable cells per ml could be detected, i.e., the limit of detection of the most-probable-number method (1). AODCs, without exception, remained approximately the same as in the original inoculum introduced into the microcosms. When the same procedures were followed, *E. coli* JM101(pUC8) entered the nonculturable state by day 21 (Table 2). ADOCs for *E. coli* JM101(pUC8) also did not change significantly.

Plasmid stability was determined by two methods: plating on TSA medium containing 100 μg of ampicillin per ml, and direct isolation of plasmid DNA from bacterial cell harvests prepared by filtration with Sterivex filters. Stability of plasmid pBR322 in *E. coli* JM83 was demonstrated, with 50% of the culturable cells in the microcosm remaining ampicillin resistant at day 4 (Table 1). The percentage of bacteria expressing the plasmid beyond day 4 could not be determined because all cells of the microcosm were nonculturable by that time. The plasmid, observed by plasmid DNA isolation, was detectable throughout the 21-day experiment (Fig. 1). Overall, the plasmid amount per cell decreased, but the plasmid remained detectable in cells in the artificial seawater microcosms.

Results of studies with plasmid pUC8 in *E. coli* JM101 were similar to those obtained with pBR322. The percentage of culturable *E. coli* JM101 expressing the ampicillin gene after 9 days was 65% (Table 2). Detection of ampicillin resistance was not determined after that time, since the culturable count dropped below 10^2 cells per ml, and detection of culturability could be achieved only by employing a liquid medium, i.e., the most-probable-number procedure. At 21 days, *E. coli* JM101 was nonculturable, yet plasmids were present up to 28 days in cells in the microcosm (Fig. 2), at which time the experiment was terminated. The plasmid band observed for extracted cells harvested by filtration from the microcosm on day 28 was equal to the band for plasmid DNA isolated on day 21, when the cells first became nonculturable.

Lysed cells in the microcosm did not contribute plasmid DNA, because after 50 ng of pure plasmid DNA was added to the Sterivex filter, no plasmid DNA was back isolated from the filter when the complete alkaline lysis procedure was used (Fig. 3). Plasmid bands were not observed in cell-free filtrates subjected to analysis for plasmid DNA.

DISCUSSION

To determine whether bacteria exposed to environmental conditions, simulated in laboratory microcosms, maintained

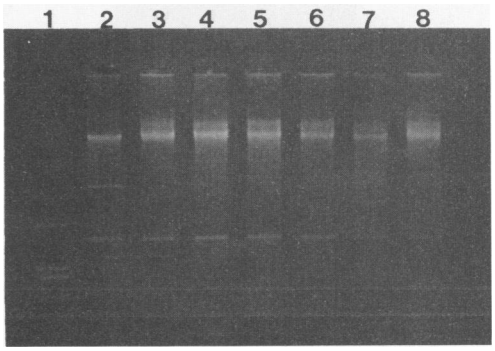

FIG. 1. Agarose gel electrophoresis of pBR322 isolated from *E. coli* JM83 after incubation in 35‰ artificial seawater at 15°C. Lane 1 contains 5 μl of molecular weight plasmid standard isolated from *E. coli* V517. Lane 2 contains 4 μl of pBR322 prepared from freshly grown cells. Lanes 3 through 8 contain 10 μl of pBR322 prepared from cells after exposure for 0, 3, 6, 11, 14, and 17 days, respectively, to artificial seawater in the microcosm.

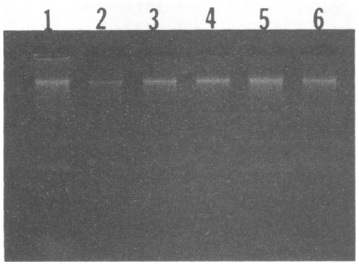

FIG. 2. Agarose gel electrophoresis of pUC8 isolated from *E. coli* JM101 after incubation in 35‰ artificial seawater at 15°C. Lane 1 contains 4 μl of pUC prepared from freshly grown cells. Lanes 2 through 6 contain 10 μl of pUC8 prepared after exposure of the host cells to artificial seawater for 14, 17, 21, 25, and 28 days, respectively, in the microcosm.

their plasmids, the ability of those cells to produce colonies on a medium containing an agent selective for that plasmid was assessed. Caldwell et al. (2) utilized such an approach to test for maintenance of plasmids in *E. coli* and observed that plasmid R388:Tn*1721* demonstrated expression in 1.06% and maintenance in 95% of the cells after starvation for 65 days in well water. When the time of exposure in the microcosm was increased to 175 days, the plasmid was not expressed when cells were plated directly on a selective medium, but when cells that were nonselectively cultured were subsequently grown under conditions of selection, 100% of the cells demonstrated maintenance of the plasmid. Flint (7) found that the plasmids R1drd-19 and R144-3 were maintained in a stable state in *E. coli* after exposure of the host cells to river water for 2 months at 15°C. Attempts by Palmer et al. (17) to isolate plasmid DNA directly from *E. coli* H10407, i.e., without culturing the cells at the end of the time of exposure to 15‰ salinity at 18°C, were not successful. In the latter case, plasmid DNA was detected when those culturable cells were transferred to brain heart infusion agar and then subjected to plasmid DNA extraction and isolation. These findings demonstrate that plasmids are indeed maintained under environmental conditions, even if they are not detected upon initial isolation of those cells remaining culturable after exposure to the environmental conditions.

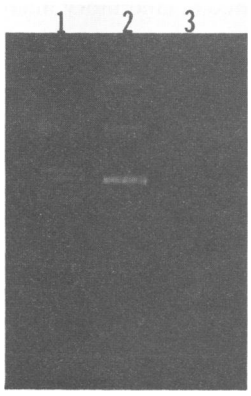

FIG. 3. Agarose gel electrophoresis of purified pUC8 DNA added to a Sterivex-GS filter unit. The extraction procedure was applied to the filter, as was done for units employed to harvest cells from the microcosm. Lane 1 contains *E. coli* V517 plasmid standards. Lane 2 contains 20 ng of commercially purified pUC8. Lane 3 shows that no plasmid DNA could be isolated from the filter after 50 ng of commercially purified pUC8 was passed through the Sterivex filter.

E. coli has been shown to enter a viable but nonculturable state after extended incubation when inoculated into microcosms simulating the estuarine or marine environment (5, 23). This state, which may be a survival stage, is an example of the so-called death phase of the growth stages of microorganisms. The death phase was reevaluated recently after methods to assess viability of these nonculturable cells, including direct viable counting (14) and microautoradiography (12), had been developed. The nonculturable stage in the life cycle of a bacterium appears to comprise a strategy for survival when the organism is exposed to conditions less than optimal for cell growth and division, such as exposure to saline environments for nonmarine bacteria.

The objective of this study was to demonstrate the maintenance of plasmids in bacteria which had entered the viable but nonculturable stage. Detection of functional plasmids in nonculturable organisms by culture methods such as those employed by Caldwell et al. (2) is not possible, since the cells, by definition, do not grow on standard media, regardless of whether a selective, nonselective, enriched, or diluted medium is used (19). However, detection of intact plasmid DNA is possible, if a large enough quantity of plasmid DNA can be extracted from cells concentrated by filtration and if the plasmid nucleic acid is isolated and purified. Since the Sterivex-GS filter allows filtration of large volumes of water, sufficient plasmid DNA can be isolated from the cells trapped on the filter, and the plasmid DNA can then be detected on agarose gels after extraction and purification. Bacteria concentrated by filtration are those retained by the 0.22-μm-pore-size filter which was employed in this study. Obviously, cells smaller than 0.22 μm are not retained, and therefore, plasmids retained in those cells can be detected if the filtrate is analyzed for plasmid DNA. Since the AODCs did not go down over the course of these experiments, no cells were lost because of cell size being less than 0.22 μm. The Sterivex filter has the additional advantage of including a filter housing, in which lysis can be accomplished (20). Palmer et al. (17), in an earlier, unsuccessful attempt to detect plasmids in nonculturable *E. coli*, used centrifugation to collect the bacterial cells for plasmid DNA isolation. The low percentage of bacteria recovered by centrifugation is the most probable explanation for the lack of success in plasmid detection by direct isolation, since very small cells are difficult to pellet (8, 20).

In the study reported here, strains of *E. coli* were exposed to artificial seawater of 35‰ salinity. These cells entered the nonculturable stage after incubation of up to 21 days at 15°C. *E. coli* JM83 maintained plasmid pBR322 for 12 days beyond culturability, i.e., the length of the incubation time of the experiment. *E. coli* JM101 was also able to maintain the plasmid pUC8 in the nonculturable stage, at approximately the same copy number as at initiation of the nonculturable state. Plasmid DNA isolated from nonculturable cells was extracted from intact bacterial cells, since AODCs did not change throughout the course of the experiment and since purified plasmid could not be back isolated from the filter, even when added directly to the filter. Thus, if it is assumed that dead bacterial cells leak nucleotides (13), the cells extracted and analyzed in this study were intact, since the plasmid nucleic acid was extracted only from cells trapped on the filter. The plasmids which were isolated were identical in characteristics to those plasmids in cells originally added to the microcosm; e.g., plasmids that were extracted from the microcosm aligned on the gels with plasmid bands

of the *E. coli* that was originally added to the microcosm. The concentration of the plasmid DNA in each sample was not determined; however, presence of the plasmid DNA provides evidence that the plasmid DNA was maintained, even though the precise amount per cell was not measured in this study. Such experiments, i.e., ones that measure the concentration of plasmid maintained per cell when the cells are nonculturable, are in progress.

The findings reported here, even though they were obtained under sterile conditions, are of significance both for public health application, e. g., analysis of water quality (1), and for introduction of genetically engineered microorganisms to the environment for bioremediation and related purposes, for treating not only water but also soil, since the latter may drain into estuarine and marine waters. Since *E. coli* has, until recently, been understood to die in seawater, maintenance of the plasmid DNA and potential for its transfer to autochthonous bacterial species cannot be discounted (15, 21). The possibility of resuscitation of the bacteria, as has been observed in downstream regrowth of *E. coli*, also cannot be discounted. If genes located on a plasmid in an organism such as *E. coli* persist in the environment long after detection of the host organism can be achieved with standard bacteriological methods of water analysis and by employing culture methods for detection, it is obvious that new methods for the bacteriological analysis of water are required. Furthermore, a reevaluation of the hypothesis that *E. coli* die in seawater is badly needed. As shown in this study, the Sterivex filter method offers a means of detecting nonculturable indicator bacteria or pathogens or both, as well as a means of tracking genetic material of genetically modified cells introduced into the environment.

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