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In Situ Survival of Plasmid-Bearing and Plasmidless *Pseudomonas aeruginosa* in Pristine Tropical Waters

NORMA E. CRUZ-CRUZ,[1] GARY A. TORANZOS,[1] DONALD G. AHEARN,[2] AND TERRY C. HAZEN[1]†*

Microbial Ecology Laboratory, Department of Biology, College of Natural Sciences, University of Puerto Rico, Río Piedras, Puerto Rico 00931,[1] and Department of Biology, University Plaza, Georgia State University, Atlanta, Georgia 30303[2]

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Two rare wild-type strains of *Pseudomonas aeruginosa* were mixed in membrane diffusion chambers and then introduced into a natural freshwater environment for 72 h. The plasmid-containing strain (R serotype 15) and the plasmidless strain (H serotype 5) had initial bacterial densities of 2×10^5 cells per ml. Samples collected from the chambers were analyzed for viable and direct counts and for acquired-resistance frequencies. Suspected transconjugant-to-donor ratios ranged from 0.5 to 1.3; transfer percentages ranged from 13 to 70%. [^{3}H]thymidine uptake indicated DNA synthesis in both strains as well as in transconjugants. These studies indicate that rare wild-type bacterial strains with large plasmid loads can survive as well as can bacteria with low plasmid loads when exposed to the in situ conditions of a tropical freshwater habitat. These results also suggest that genetic modification of indigenous microbiota through conjugation or transformation is feasible when rare wild-type strains or genetically engineered microorganisms are released in large numbers in tropical aquatic ecosystems.

To properly assess the risks of releasing genetically engineered microorganisms into the environment, research needs to be performed on the survival, establishment, growth, and transfer of genetic information of model bacteria. Plasmid-containing wild-type bacterial strains have been used as model organisms for the required genetically engineered microorganism research (6). Their survival and plasmid-transfer abilities in the natural environment depend primarily on the selective advantage that their DNA can confer, namely to resist harsh environmental conditions (4, 10). Some studies have shown that plasmid-bearing bacteria usually cannot compete effectively against the indigenous, plasmidless gut microbiota of mammals unless the latter indigenous microbiota are repressed (19). Debilitated *Escherichia coli* strains have also been selected for in situ and in vivo survival and gene transfer studies to minimize their escape and establishment in the environment. However, there is little information on the survival and genetic aspects of bacteria other than *E. coli* (20) in the environment.

The purpose of this study was to compare the in situ survival rates of two wild-type bacterial strains, a plasmid-bearing strain and a plasmidless strain, by using diffusion chambers. A plasmid-bearing strain of *Pseudomonas aeruginosa* was used as a model for genetically engineered strains. The plasmids in the wild-type strain are nonessential for the survival of this strain (15). Since they are not required for survival, these exogenous pieces of DNA could generate a growth stress in their host cells; this may give other bacteria a competitive disadvantage. The idea that plasmids are a biosynthetic burden upon their host cells comes from studies in which cells exhibited an enhanced growth rate upon loss of their plasmids (4, 10).

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The study site was a pristine freshwater stream in the Mameyes River watershed located on the northeastern coast of the island of Puerto Rico at 18° 15′ N, 65° 45′ W. This watershed has a drainage area of 27.27 km^2 and a total length of 17.1 km. The annual average precipitation in the upper third of the estuary is 395 cm; this area is classified as a cloud rain forest and is part of the Luquillo Experimental Forest of the U.S. Forest Service. Detailed descriptions of the study area have been provided by Carrillo et al. (3).

A Hydrolab Surveyor, model 4041 (Hydrolab Corp., Austin, Tex.), was used to monitor pH and air and water temperature in situ. Water samples were analyzed for dissolved oxygen by the Winkler method (2). Alkalinity and hardness were measured in the field by using a Mini Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Other samples were preserved (mercuric chloride, sulfuric acid, or zinc acetate) for further analysis in the laboratory for nitrates plus nitrites, sulfates, phosphates, and total phosphorus as recommended in *Standard Methods for the Examination of Water and Wastewater* (2).

Two rare wild-type strains of *P. aeruginosa*, R and H, were used in this study. The R strain (serotype 15, harboring one 100-megadalton plasmid and two 5- to 8-megadalton plasmids) is an oil-and-water-emulsion isolate (for plasmid profiles, etc., see reference 14). This strain is resistant to 0.12% Germall 115 (Sutton Laboratories), which is an effective preservative against gram-negative bacteria used in the cosmetic industry (5). The *P. aeruginosa* H strain (serotype 5, harboring no plasmids) is an isolate from a corneal ulcer (15) and is sensitive to Germall 115 (D.G.A., unpublished data). Both serotypes grow readily on all laboratory media. Their plasmid profiles are apparently stable, and antibiotic sensitivity patterns are quite similar. Both strains are resistant to ampicillin, cefamandole, cephalothin, neomycin, streptomycin, tetracycline, and triple sulfa. Both strains are sensitive to amikacin, carbenicillin, gentamicin, mezlocillin, polymyxin, and tobramycin. The resistance to Germall

* Corresponding author.
† Present address: Environmental Sciences Division, Savannah River Laboratory, E. I. du Pont de Nemours & Company, Inc., Aiken, SC 29808.

TABLE 1. Water quality parameters

Time (h)	Value for the following parameter[a]:							
	DO (mg/liter)	PO_4 (mg/liter)	TP (mg/liter)	SO_4, 1,000 mg/liter	NO_{2+3} (mg/liter)	ATEMP (°C)	WTEMP (°C)	pH
0	4	0.008	0.035	0.025	0.095	21	20	4.7
24	5	ND	0.025	ND	0.160	19	19	4.2
48	7	0.008	0.040	0.005	0.040	19	18	5.6
72	5.3	ND	0.030	ND	0.035	19	19	5.9
Mean ± SE	5.3 ± 0.6	0.008 ± 0	0.033 ± 0.003	0.008 ± 0.006	0.083 ± 0.029	19.5 ± 0.5	19.0 ± 0.7	5.09 ± 0.39

[a] ND, Not detectable; ATEMP, air temperature; WTEMP, water temperature; DO, dissolved oxygen; SO_4, sulfates; NO_{2+3}, nitrites plus nitrates; TP, total phosphorus.

exhibited by the serotype 15 R strain can be lost in culture and has not yet been demonstrated to occur within the plasmids of this strain; however, the resistance to Germall can be transferred to the serotype 5 H strain.

Pure cultures of the *P. aeruginosa* R and H strains were grown in nutrient broth at 37°C for 24 h. The cells were harvested by centrifugation and washed in filter-sterilized (0.2-μm-pore-size filter) phosphate-buffered saline (pH 7). The number of cells per milliliter was determined with a ZM Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) and adjusted to 10^5. Ninety milliliters of *P. aeruginosa* R and 10 ml of *P. aeruginosa* H were mixed and introduced into 100-ml sterile diffusion chambers through 0.2-μm-pore-size filters before immersion at the study sites. The chambers and their use were described before (11, 12, 23).

Mixed wild-type bacterial strains were placed in four chambers at the site. Each chamber was suspended 0.25 to 1 m below the surface, and samples (1 ml) were taken with sterile syringes at different time intervals for 5 days. Half of each sample was fixed with 1.5 ml of 10% phosphate-buffered Formalin (pH 7) and refrigerated for later enumeration with a Coulter Counter (12). Microautoradiography was performed to measure uptake-active microorganisms by using [^{3}H]thymidine (22). Additional samples (1 ml) were collected at the same time, incubated with 0.01 ml of [^{3}H]thymidine (10 mCi/mmol) (Du Pont, NEN Research Products, Boston, Mass.) at an in situ temperature for 2 to 2.5 h on a shaker, and fixed with 0.01 ml of formaldehyde (37%). Samples were kept on ice and transferred to the laboratory, where they were analyzed. The indirect-fluorescent-antibody method was combined with the microautoradiography technique of Tabor and Neihof (FAMA; reference 22) to determine total cell counts and percent of bacteria active in DNA synthesis.

For in situ acquired-resistance studies, 1-ml samples were collected from the chambers as described above and spread on nutrient agar plates. After 24 h of incubation at 37°C, the colonies were replica plated onto nutrient agar plates containing Germall 115. After 24 h, the colonies that grew were serotyped by slide agglutination with the *P. aeruginosa* Antiserum Set (Thomas Scientific, Swedesboro, N.J.). Acquired-resistance frequencies were calculated as the ratio of resistant recipient bacteria to resistant donor bacteria.

Statistical tests were done with programs developed for the Macintosh computer. Relationships between parameters measured were determined with multiple correlation and regression analyses. Data that were heteroscedastic, as determined by measurements of skew and kurtosis, were transformed to make them more homoscedastic by using either $\log_{10}(x + 1)$ or arcsine square root. Probabilities less than or equal to 0.05 were considered significant (24).

In general, this study showed that both plasmid-containing and plasmidless *P. aeruginosa* strains could survive well in a tropical freshwater ecosystem (average bacterial density was at least 10^4 cells per ml for all viable and direct enumeration methods) with a water temperature of 19 to 20°C and a pH of 4.2 to 5.85. Representative water quality data are given in Table 1. Viable counts in the chambers (Fig. 1) increased nearly 2 orders of magnitude during the first 6 h and, overall, increased significantly over time ($t = 3$, df = 6, $P < 0.01$). Coulter Counter densities for total bacteria also increased after 24 h and showed a similar pattern. Viable count densities, however, were 1 to 2 orders of magnitude higher than Coulter Counter densities; this was undoubtedly due to cell clumping or the large aperture size (10 μm) of the Coulter Counter used.

Immunofluorescence-determined densities of the two serotypes showed that the increases in viable density and Coulter Counter density were due to increases in the density of serotype 5 and not of serotype 15 (Fig. 2). The density of serotype 5 increased significantly over time ($t = 2.5$, df = 6, $P < 0.05$), while no significant change in density was observed for serotype 15. Although incorporation of [^{3}H]thymidine appeared to be quite low (Fig. 2), it is typical for bacteria in natural environments (22). [^{3}H]thymidine incorporation was significantly higher for serotype 5 during the first 24 h ($t = 2.3$, df = 8, $P < 0.05$). Thus, uptake of [^{3}H]thymidine also showed that serotype 5 was actively growing while serotype 15 was remaining static. Since serotype 5 started out at 10% of the density of serotype 15, this

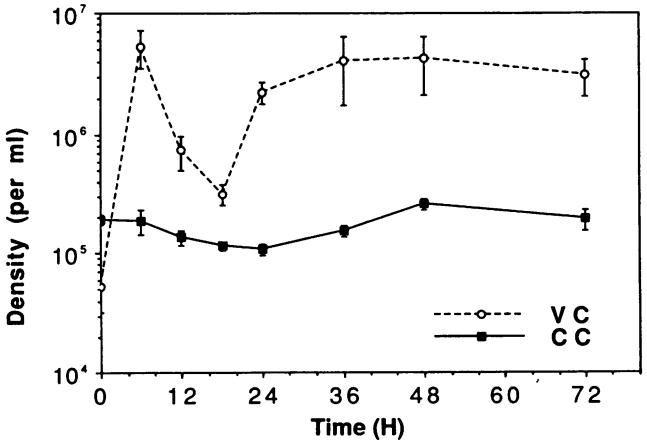

FIG. 1. Survival of *P. aeruginosa* in the Mameyes River. Densities were determined by using the Coulter Counter (CC; cells per milliliter) and by total viable count (VC; CFU per milliliter) (mean ± 1 standard error; $n = 4$).

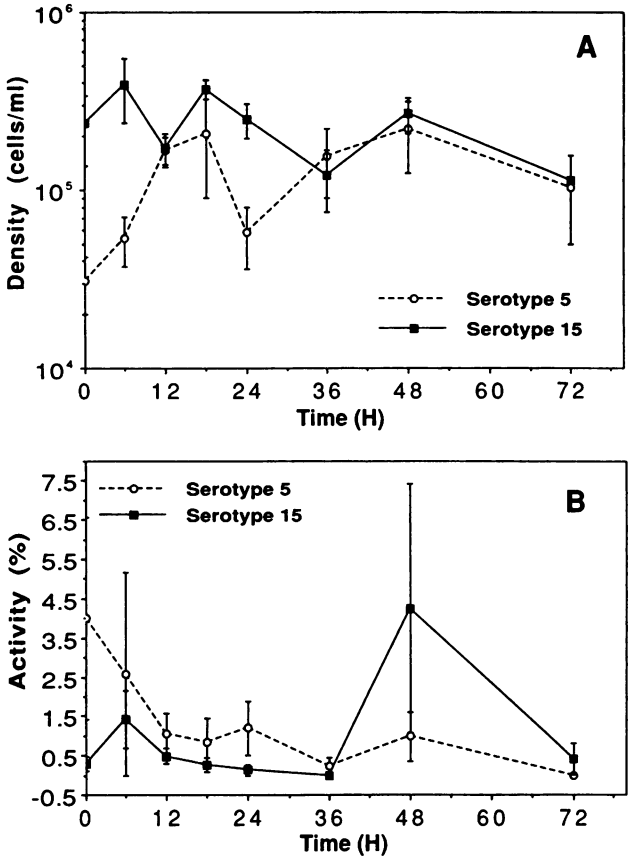

FIG. 2. Survival densities, determined by immunofluorescence (A), and activity, determined by microautoradiographs (B), of *P. aeruginosa* serotypes 5 and 15 in the Mameyes River (mean ± 1 standard error; $n = 4$).

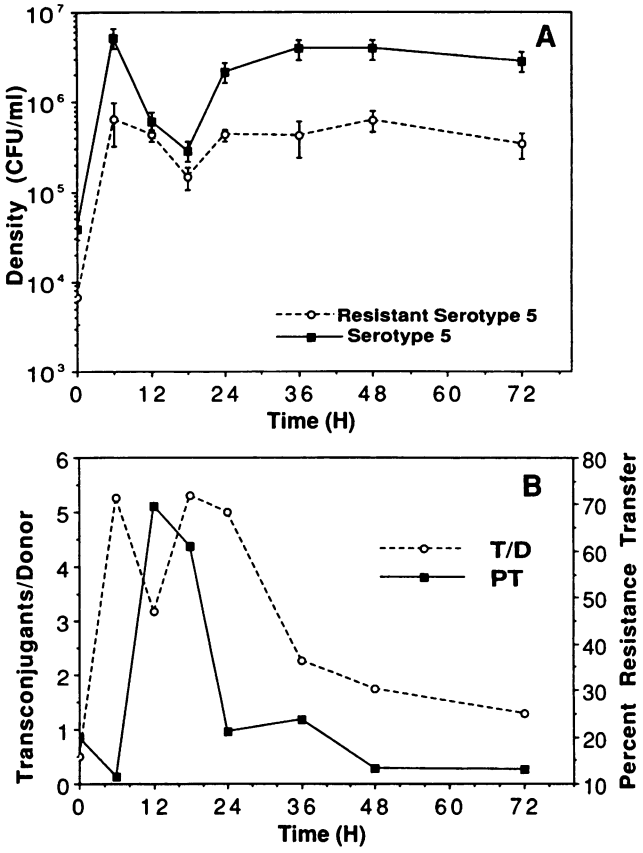

FIG. 3. Survival of *P. aeruginosa* serotype 5 and suspected transconjugants (serotype 5 with acquired resistance to Germall 115) (A) and changes in the ratio of suspected transconjugants (serotype 5 with acquired resistance to Germall 115) to donor cells (serotype 15) and the percentage of serotype 5 cells with resistance to Germall 115 (percent resistance transfer) (B) in the Mameyes River (mean ± 1 standard error; $n = 4$).

implies that the carrying capacity of the environment was apparently the same for both serotypes. Even though serotype 5 was not carrying a heavy plasmid load, as serotype 15 was, the density of serotype 5 increased only to the same relative density as that of serotype 15, i.e., 5.0×10^5 cells per ml.

Thus, the presence of plasmids in *P. aeruginosa* serotype 15 did not confer a selective disadvantage upon this bacterium. Grabow et al. (8) did not observe significant survival differences between plasmid-bearing and plasmidless host cells in natural waters. Devanas et al. (6) obtained good survivability for plasmid-containing bacteria in nutrient-rich sediment. However, Smith et al. (20) have observed good survivability of *E. coli* R plasmid-carrying strains in low-nutrient seawaters. These studies indicate that plasmid-bearing bacterial strains can survive as well as their plasmidless counterparts when exposed to the in situ conditions of any high-nutrient freshwater habitat.

The density of suspected transconjugants (Germall resistant, serotype 5) increased 2 orders of magnitude during the first 6 h (Fig. 3). This increase directly paralleled the increase in density of recipient *P. aeruginosa* (Germall sensitive, serotype 5). The density of suspected transconjugants declined after 6 h, even though the density of recipients had not changed. The initial donor-to-recipient ratio was 9:1, clearly favoring rapid formation of transconjugants; however, after only 6 h the ratio was only 4:1, and at 12 h the ratio was nearly equal (Fig. 2). Thus, the number of transconjugants per donor declined slightly as the recipients grew rapidly, even though the percentage of resistance transfer increased up to 70% (Fig. 3). As recipient density leveled off and the donor-to-recipient ratio became less favorable at 12 h, the percentage of plasmid transfer increased and the number of suspected transconjugants per donor decreased (Fig. 3). Both percent resistance transfer and the ratio of suspected transconjugants to donors declined as the density of recipient and donor bacteria stabilized and the density of suspected transconjugants came into equilibrium with these densities.

The resistance transfer frequency (suspected transconjugants per donor; T/D) results (0.5 to 1.3 T/D) were higher than the in situ T/D values obtained in other membrane diffusion chamber studies performed in raw sewage (3.2×10^{-5} to 1.0×10^{-6} [1]), sterile sewage (5.9×10^{-5} [13]), and sterile pond water (4.4×10^{-7} to 4.7×10^{-8} [7]). A study with high T/D values that used microcosms with lake water, *P. aeruginosa* strains, and bacteriophages had a frequency of transduction of 5×10^{-2} to 25×10^{-2} when a 20:1 donor-to-recipient ratio was used (18). As in the present study, Saye et al. (18) found that the concentration of plasmid-containing donor cells significantly influences the yield of detectable transconjugants. High transfer frequencies of 0.8 to 1.6 have also been reported in backcrosses of transconjugants to *E. coli* K-12 F^- recipients performed in

flasks containing Penassay broth (19). In this study, the recipient population (serotype 5) grew during the first few hours of exposure and both serotype populations actively synthesized DNA. This is consistent with the studies of Altherr and Kasweck (1) and of Gowland and Slater (7), which suggested that low transfer frequencies were a reflection of a stressful or nutrient-lacking environment.

Extensive data have been collected over the years concerning the microbiological background flora and the physical and chemical attributes of the study site in the Mameyes River (11). Even though this site is relatively nutrient poor, these studies have demonstrated that *E. coli*, *Klebsiella pneumoniae*, and *Candida albicans* can survive and remain active for much longer periods of time then they do in temperate waters (3, 11, 16, 23). Recently, we have even demonstrated that *E. coli* at this site may be of autochthonous origin (9, 17). This rain forest watershed and other pristine freshwater sites in Puerto Rico typically have high numbers of coliforms and fecal coliforms in the complete absence of any human or animal fecal contamination (17). The normal flora of these environments is typically mesophilic, a finding that is not surprising since water temperatures never fall below 18°C and usually range from 25 to 35°C (17). Thus, the environmental conditions in tropical fresh waters are very receptive to establishment and growth of mesophilic, thermotolerant bacteria.

Few studies have addressed the survival and transfer of bacterial genetic information in situ, and none have been performed in the tropics. Most of the work concerning these organisms had been performed under controlled conditions in the laboratory which do not necessarily apply to in situ genetic recombinations and bacterial persistence. The present study suggests both survival and mobilization of new genetic attributes from novel wild-type *P. aeruginosa* strains under in situ environmental conditions in tropical fresh waters.

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