

Multiple Antibiotic Resistant *Escherichia coli* from a Tropical Rain Forest Stream in Puerto Rico*

C. E. CARRASCO,¹ H. J. ALVAREZ,² N. ORTIZ³, M. BISBAL,³ W. ARIAS,⁴
J. W. SANTO DOMINGO,⁵ AND T. C. HAZEN^{6**}

¹Department of Biology, University of Puerto Rico-Ponce, Ponce, Puerto Rico 00732

²Department of Pedagogy, University of Puerto Rico, Río Piedras, Puerto Rico 00931

³Department of Biology, University of Puerto Rico-Humacao, Humacao, Puerto Rico 00791

⁴Department of Biology, InterAmerican University, Río Piedras, Puerto Rico 00919

⁵Westinghouse Savannah River Company, Environmental Sciences and Biotechnology Section, 704-8T,
Aiken, South Carolina 29808-0001

⁶Department of Biology, College of Natural Sciences, University of Puerto Rico Río Piedras, Puerto Rico 00931

ABSTRACT.—The resistance to antibiotics and presence of plasmids in fecal coliforms isolated from the tropical rain forest of El Yunque, Puerto Rico, was studied. Densities of fecal coliforms obtained from a pristine site and a sewage contaminated site in this forest's watershed were higher than maximum levels allowed for recreational waters. Approximately 70% of the fecal coliform isolates were identified as *Escherichia coli*. Multiple antibiotic resistance (MAR) was common for isolates at both sites; however, the site receiving sewage effluent had a greater proportion of MAR isolates. Antibiotic resistance (R) plasmids were recovered from MAR isolates of each site. All recovered plasmids were approximately 1 kilobase. The recovered plasmids seemed capable of transforming *E. coli* HB101 in vitro. The high concentrations of enteric bacteria, R plasmid mobility, and documented long term survival of fecal bacteria in tropical freshwater environments give increasing importance to adequate sewage treatment and to better methods to monitor bacterial indicators of fecal contamination for tropical areas.

RESUMEN.—Fue estudiada la resistencia a antibióticos y la presencia de plásmidos en coliformes fecales aislados del bosque tropical húmedo El Yunque en Puerto Rico. Las densidades de coliformes fecales obtenidas en un lugar pristino y en un lugar expuesto a contaminación de aguas residuales en la cuenca de un río en este bosque fueron más altas que las permitidas por regulaciones de calidad de aguas recreacionales. Aproximadamente 70% de las cepas de coliformes fecales aisladas fueron identificadas como *Escherichia coli*. Resistencia múltiple hacia antibióticos fue detectada en cepas aisladas de ambos lugares, aunque la proporción de cepas con resistencia multiple fue más alta en las bacterias aisladas del lugar con historia de contaminación. Plásmidos que confieren resistencia a antibióticos fueron extraídos de varias de las cepas aisladas. El tamaño de estos plásmidos fue de aproximadamente 1 kilobase. Además, estos plásmidos parecen poder transformar a *E. coli* HB101 in vitro. Las concentraciones altas de bacterias entéricas, la movilidad de plásmidos, y la prolongada sobrevivencia de bacterias fecales en ambientes tropicales de agua dulce enfatizan aún más la importancia del tratamiento adecuado de aguas residuales y la necesidad de mejores métodos para monitorear bacterias indicadoras de contaminación fecal en áreas tropicales.

INTRODUCTION

Antibiotic resistance genes in most bacteria are frequently found in extrachromosomal elements known as R plasmids. Antibiotic resistant bacteria are widespread, as illustrated by the number of environments from which they have been isolated (Goyal et al., 1979; Niemi et al., 1983; Shaw and

Cabelli, 1980). Certain habitats seem to carry high densities of bacteria containing R plasmids (e.g., long term sedimentation pools in treatment plants; Bell et al., 1983). These R plasmids may be transferred to pathogenic bacteria via conjugation, transformation, or viral transduction (Grabow et al., 1975; Mach and Grimes, 1982).

The presence of indigenous bacteria that harbor R plasmids in recreational and drinking water sources could pose a serious health hazard due to their potential resistance to normal antibiotic treatments. This

* This paper is dedicated to the memory of Dr. José Ramón Ortiz.

** Corresponding author.

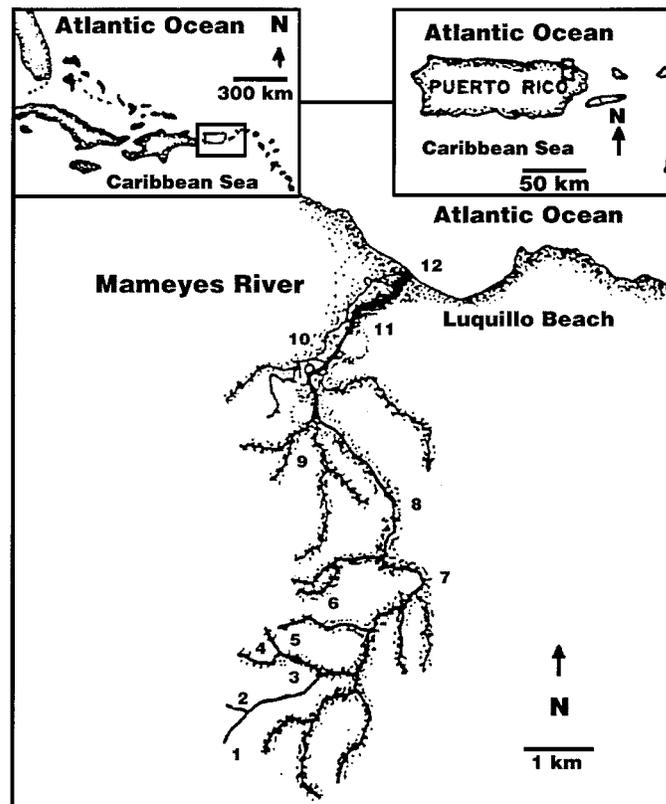


FIG. 1. Map of study sites in Mameyes River. Samples used in this study were collected from site 1 and 9.

health risk is enhanced when potentially pathogenic bacteria survive for prolonged periods in aquatic environments, as indicated by the high densities of coliform bacteria in tropical waters in the absence of known human fecal sources (Hazen et al., 1988; Oluwande et al., 1983; Santiago-Mercado and Hazen, 1987). For example, *Escherichia coli* strains have been isolated 20 m above the ground from rain forest epiphytic plants (Bermúdez and Hazen, 1988). Moreover, several studies have shown that *E. coli* can survive for extended periods in tropical waters (Carrillo et al., 1985; Hazen et al., 1988; Santo Domingo et al., 1989) and may even be indigenous in tropical rain forests (Bermúdez and Hazen, 1988). More importantly, some of these environmental strains have been found to resist several antibiotics (Rivera et al., 1988). Thus, it is plausible that plasmid mobilization in *E. coli* might occur in some of these unusual habitats.

This study examined the occurrence of multiple antibiotic resistance (MAR) plasmids in *E. coli* at a contaminated and at a pristine site in a tropical rain forest watershed. These plasmids were isolated and tested for their ability to be transferred to a plasmidless *E. coli* strain.

MATERIALS AND METHODS

Study Site.—The Mameyes River (Fig. 1) is located in the northeast section of Puerto Rico. The river originates in a cloud rain forest in a pristine portion of the Luquillo Experimental Forest. Samples were taken from two sites. Site 1 is a pristine area in the rain forest, representing the highest study site in the watershed, while Site 9 is several km downstream, just below the outfall from a primary sewage treatment plant (López-Torres et al., 1987). These sites were selected to represent the extremes of water quality found in this watershed. For a more

complete description of the Mameyes River watershed and sites 1 and 9, see Carrillo et al. (1985).

Microbiological Methods.—Water samples were collected slightly below the surface of running and still waters with sterile 180 ml Whirl-Pak bags (Nasco, Ft. Wilkinson, WI). Samples were brought to the laboratory at ambient temperature and analyzed within 3 h of collection. Appropriate volumes (i.e., 0.1–10 ml) were filtered through 0.45 μ m pore size, 47 mm diameter membrane filters (GN-6, Gelman Instrument Co., Ann Arbor, Mich.). Each filter was incubated at $44.5 \pm 0.1^\circ\text{C}$ for 24 h on mFC agar (Difco Laboratories, Detroit, Mich.). Dark blue colonies were considered fecal coliform positive (Santiago-Mercado and Hazen, 1987). Random isolates were then streaked on trypticase soy agar (Difco) and incubated at 37°C for 24 h.

Colonies were characterized by Gram staining reaction and API 20E strips (Analytab Products, Plainview, NY). Antibiotic resistance was assayed on Mueller-Hinton agar (Difco), using antimicrobial discs (Sigma Chemical Co., St. Louis, MO) with ampicillin (Amp), chloramphenicol (C), penicillin (Ps), sulfathiazol (Su), tetracycline (Te), rifampicin (Ra), and streptomycin (St). Susceptibility was determined according to the National Committee for Clinical Laboratory Standards, Approved Standards ASM2 (APHA, 1985).

Plasmid Extraction.—The presence of plasmids was investigated in the *E. coli* isolates EC1 and EC7 from site 1 (Table 1) and in the strains EC14 and EC15 from site 9, since their multiple antibiotic resistance suggested the possible presence of R plasmids. Plasmid DNA was isolated according to Dillon et al. (1985). Briefly, bacterial cells were grown overnight in trypticase soy broth (TSB, Difco) at 37°C . Plasmids were amplified through the addition of 150 $\mu\text{g}/\text{ml}$ chloramphenicol (Sigma) to mid log cells and further incubated for 3–5 h. The cells were harvested by centrifugation at $10,730 \times g$ for 15 min at 4°C . The pellet was digested with lysozyme (10 mg/ml) in TES buffer (30 mM Tris HCl, pH 8, 50 mM EDTA, 5 mM NaCl) and 20% Triton X-100 (Sigma). The lysate was centrifuged at

TABLE 1. Antibiotic sensitivity of fecal coliform environmental isolates.

Iso- lates	Antibiotics						
	C	Am	Ps	Su	Te	Ra	St
EC1	–	+	+	+	–	ND	ND
EC2	–	–	+	–	–	ND	ND
EC3	–	–	+	–	–	ND	ND
EC4	–	–	+	–	–	ND	ND
EC5	+	–	+	–	–	ND	ND
EC6	–	–	+	–	–	ND	ND
EC7	–	–	+	–	–	ND	ND
EC8	–	+	+	–	–	ND	ND
EC9	–	+	+	–	–	ND	ND
EC10	–	–	+	–	–	ND	ND
EC11	–	–	+	–	–	ND	ND
EC12	–	+	+	–	–	+	+
EC13	–	+	+	–	–	ND	ND
EC14	–	+	+	+	+	ND	ND
EC15	–	+	+	+	–	ND	+
EC16	–	+	+	–	–	ND	ND
EC17	–	–	+	–	–	ND	ND
EC18	–	–	–	–	–	ND	ND

C = Chloramphenicol; Am = Ampicillin; Ps = Penicillin; Su = Sulfathiazol; Te = Tetracycline; Ra = Rifampicin; St = Streptomycin; + = Resistant; – = Sensitive; ND = not determined; Isolates EC1–EC9 from site 1, EC10–EC18 from site 9.

$19,000 \times g$ for 20 min at 4°C . The supernatant was adjusted to 4 ml with TES buffer and 3.65 g CsCl_2 (Sigma) was added and gently dissolved into the solution. Ethidium bromide (EtBr; 250 $\mu\text{g}/\text{ml}$, final concentration) was then added and mixed gently. This final mixture was centrifuged in ultraclear tubes at 44,000 rpm at 5°C for 42 h, using a type 50 rotor, and a refrigerated ultracentrifuge (Beckman).

The plasmid band was extracted and washed several times with aqueous isobutanol to remove the ethidium bromide (Maniatis et al., 1982). Plasmid DNA was then precipitated with 2.5 vol 95% ethanol and 300 mM sodium acetate (final concentration) and stored at -20°C . The precipitate was centrifuged at full speed in a microcentrifuge (Eppendorf) and the pellet was air dried. The DNA was dissolved in 1 ml TE buffer (50 mM Tris HCl, pH 8 / 20 mM EDTA) and dialyzed for 24 h, as described in Maniatis et al. (1982). Sample concentration was determined using absorption at 254

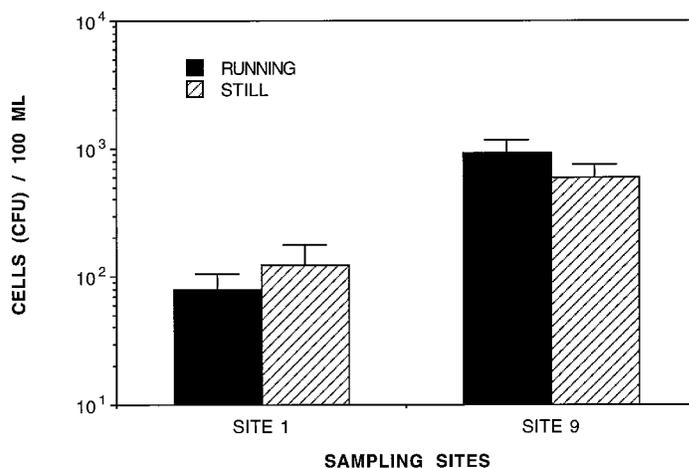


FIG. 2. Density of fecal coliforms by site in the Mameyes River. Samples were taken from still (calm) and running waters. Values represent means of triplicates and bars represent standard deviation of mean.

nm (1 OD = 50 μ g/ml of double stranded DNA). Subsamples were subjected to electrophoresis in a Bio Rad mini Sub DNA Cell through a 0.5% agarose gel at 6 v for 15 h at room temperature in 1 \times TBE (89 mM Tris / 89 mM boric acid / 2 mM EDTA, pH 8) buffer. Whole lambda phage (λ) DNA (48 kb) and λ DNA Hind III digests (23.1, 9.4, 6.7, 4.4, 2.3, 2.0, and 0.56 kb fragments, BRL) were used as markers.

Transformation Experiments.—The plasmid extracted from strain EC14 was chosen to transform *E. coli* HB101. Recipient HB101 cells were made competent with CaCl_2 following Maniatis et al. (1982). HB101 cells were grown in Luria broth (LB) at 37°C for 2–4 h with vigorous shaking, chilled for 10 min on ice and centrifuged at 4,000 \times g for 5 min at 4°C. The cells were resuspended in 50 ml of an ice-cold (ca. 0°C) solution of 50 mM CaCl_2 – 10 mM Tris HCl (pH 8), chilled for 15 min and centrifuged as before. Cells were resuspended again in 7 ml of the CaCl_2 / Tris HCl solution, dispensed in 200 μ l aliquots into prechilled tubes, and stored for 24 h at 4°C. The plasmid DNA (40 ng / 100 μ l) from strain EC14 was added in TE buffer to competent HB101 cells mixed and stored on ice for 30 min. The mixture was heat shocked at 42°C for 2 min and incubated at 37°C for 30 min on 1 ml of LB. Transformed cells were tested for tet-

racycline resistance on LBA plates (LB + 15 g noble agar / l).

RESULTS AND DISCUSSION

The density of fecal coliforms at both sites was on average equal or higher than 80 CFU/100 ml (Fig. 2), which is above the maximum levels allowed for recreational waters (Cabelli et al., 1983). However, such densities are commonly found in rivers and other pristine aquatic habitats throughout Puerto Rico, regardless of obvious sources of fecal or industrial contamination (Santiago-Mercado and Hazen, 1987). Densities of fecal coliform bacteria were considerably higher at site 9 (the sewage contaminated site). Previous studies have shown a long survival time for *E. coli* in these waters at both sites (Carrillo et al., 1985; López-Torres et al., 1987), possibly explaining such high densities of coliform bacteria. Approximately 70% of the positive fecal coliform isolates were confirmed as *E. coli*, which is considerably lower than for temperate waters. This low percentage of *E. coli* strains among the fecal coliform isolates is typical for tropical waters, and probably is due to the dominance of mesophilic background flora in such waters (Rivera et al., 1988; Santiago-Mercado and Hazen, 1987). Previous studies in this watershed have demonstrated that *E. coli* isolated from epiphytes in

trees 20 m above the ground has the same genomic G+C content as the clinical strain *E. coli* B (Bermúdez and Hazen, 1988). These rain forest isolates were shown to have at least 70% DNA homology with *E. coli* B, a bona fide fecal coliform. Thus, as suggested by Hazen (1988), *E. coli* appears to be indigenous to many tropical waters and should not be considered an appropriate indicator of recent human fecal contamination.

Most isolates (94%), including all the fecal coliforms isolated from the highest point in the watershed (site 1), showed resistance to penicillin (Table 1). In contrast, most isolates were sensitive to tetracycline (94%), chloramphenicol (94%), and sulfathiazol (83%). The percentage of environmental *E. coli* strains which were resistant to two or more antibiotics (53%) was higher than the range previously reported (Grabow et al., 1975; Kelch and Lee 1978; Smith, 1970). Multiple antibiotic resistance (MAR) to at least three antibiotics was most common in bacteria from site 9 (17.6%) when compared to site 1 (6%) (Table 1).

The plasmids in strains EC1, EC7, EC 14, and EC15 were approximately 1 kilobase (kb), according to their mobility in agarose gel. Kobori et al. (1984) detected a plasmid with a molecular weight of ca. 1.5 kb and suggested that this may be the smallest plasmid so far reported in natural environments. Our study showed that small plasmids are present in environmental isolates of *E. coli* and furthermore suggests that plasmids as small as 1 kb may be common among these tropical bacterial strains. A wide range of plasmid sizes have been reported for different bacteria (Helinski 1973). Since Hanahan (1983) has demonstrated that the probability of transformation increases linearly with decreasing plasmid size, these small plasmids may have a very high transforming potential. Glassman and McMicol (1981) suggested that there is a preponderance of small plasmids in isolates from clean sites and a preponderance of larger ones in isolates from polluted sites. The isolation of small plasmids in our study would suggest that both sites were unpolluted. However, small plasmids were also found in strains isolated from site 9,

which is the only site in the watershed known to receive sewage contamination, as supported by the higher concentrations of phosphorus and nitrogen observed in this site when compared to other study sites (Carrillo et al., 1985; Elías-Montalvo et al., 1988). Thus, our results suggest that the isolation of small plasmids from fecal coliforms (i.e., *E. coli*) could not be used to indicate previous exposure to fecal contamination.

Although we did not confirm plasmid transformation using molecular methods, we based our conclusions on the acquisition of tetracycline resistance by *E. coli* HB101, which is sensitive to this antibiotic. Resistance to tetracycline was transferred in all cases, and was clearly evident after 24 h in those cultures where at least 100 μ l of the mixture (HB101 plus Te-R-plasmid DNA) was plated. Where smaller volumes were used (20 to 75 μ l), growth was not evident at 24 h, although a few colonies were obtained by 72 to 96 h. The controls showed that HB101 control cells did not grow on the LBA-Te medium.

Gram negative bacteria have evolved mechanisms which favor the development of competent cells under conditions which inhibit cell division. For example, transformable *Acinetobacter* strains become competent when entering a stationary phase (Juni, 1972). Although homologous DNA is preferentially transferred in gram negative bacteria, there is evidence that transformation also occurs by uptake of non-homologous DNA via broad range host plasmids (Smith et al., 1981). Recent studies by Cruz-Cruz et al. (1988) have shown that in situ genetic transfer can take place between *Pseudomonas aeruginosa* strains at pristine tropical waters. These studies showed that transfer occurred within 3 h, and that transconjugants did not decrease in survivability.

Considering that even under stressed conditions *E. coli* could survive in appreciable densities for 3 h, it is likely that R plasmids that reach tropical waters through *E. coli* could transform pathogenic *E. coli* strains as well as other indigenous microbiota. Shaw and Cabelli (1980) reported an outbreak of recreational waterborne enteric

disease caused by antibiotic resistant *Shigella* spp., while other reports have associated a number of deaths in several parts of the world due to MAR containing bacteria (Baine et al., 1977; Gangarosa et al., 1972). The fact that antibiotic resistance may be readily transferred among the genera of Enterobacteriaceae has been studied extensively (Bell et al., 1983; Hanahan, 1983). This study suggests that tropical waters exposed to sewage contamination may present an even greater danger to public health due to the genetic transfer of R plasmids and the long survival of *E. coli* in these environments. Adequate indicators of recent human fecal contamination for monitoring water contamination in the tropics are desperately needed to increase the health of the 65% of the world's population that lives in tropical areas.

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LITERATURE CITED

- APHA (American Public Health Association). 1985. Standard Methods for the Examination of Water and Wastewater. 16th ed. American Public Health Association, Washington, D.C., 1467 pp.
- Baine, W. B., J. J. Farmer, E. J. Gangarosa, G. T. Herman, C. Thornberry, and P. A. Rice. 1977. The typhoid fever in the United States associated with the 1972-1973 epidemic in Mexico. *J. Infect. Diseases* 135: 649-653.
- Bell, J. B., G. E. Elliott, and D. W. Smith. 1983. Influence of sewage treatment and urbanization on selection of multiple resistance in fecal coliform populations. *Appl. Environ. Microbiol.* 46:227-232.
- Bermúdez, M. and T. C. Hazen. 1988. Phenotypic and genotypic comparison of *Escherichia coli* from pristine tropical waters. *Appl. Environ. Microbiol.* 54: 979-983.
- Cabelli, V. J., A. P. Dufuor, L. J. McCabe, and M. A. Levin. 1983. A marine recreational water quality criterion consistent with indicator concepts and risk analysis. *J. Water Pollut. Control Fed.* 55:1306-1314.
- Carrillo, M., E. Estrada, and T. C. Hazen. 1985. Survival and enumeration of the fecal indicators *Bifidobacterium adolescentis* and *Escherichia coli* in a tropical rain forest watershed. *Appl. Environ. Microbiol.* 50:468-476.
- Cruz-Cruz, N. E., G. A. Toranzos, D. G. Ahearn, and T. C. Hazen. 1988. In situ survival of plasmid and plasmid-less *Pseudomonas aeruginosa* in pristine tropical waters. *Appl. Environ. Microbiol.* 54:2574-2577.
- Dillon, J. R., A. Nasim, and E. R. Nestmann. 1985. Recombinant DNA Methodology. pp. 3-23. Wiley Press, New York.
- Elías-Montalvo, E. E., A. Calvo, and T. C. Hazen. 1989. Survival and distribution of *Yersinia enterocolitica* in a tropical rain forest stream. *Curr. Microbiol.* 18: 119-126.
- Gangarosa, E. J., J. V. Bennett, C. Wyatt, P. E. Pierce, J. Olarte, P. M. Hernandez, V. Vazquez, and P. Bes-sudo. 1972. An epidemic associated episome. *J. Infect. Diseases* 126:215-218.
- Glassman, D. L. and L. A. McMicol. 1981. Plasmid frequency in natural population estuarine microorganisms. *Plasmid* 5:231.
- Goyal, S. M., C. P. Gerba, and J. L. Melnick. 1979. Transferable drug resistance in bacteria of coastal coral canal waters and sediment. *Water Res.* 13: 349-356.
- Grabow, W. K. O., W. O. Prozesky, and J. S. Burger. 1975. Behavior in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. *Water Res.* 9:777-782.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
- Hazen, T. C. 1988. What do fecal coliforms indicate in tropical waters? *Inter. J. Tox. Assessment* 3:461-477.
- Hazen, T. C., F. A. Fuentes, and J. W. Santo Domingo. 1988. In situ survival and activity of pathogens and their indicators. In *Recent Advances in Microbial Ecology: Proceedings of the 4th International Symposium of Microbial Ecology*, pp. 406-411.
- Helinski, D. R. 1973. Plasmid determined resistance to antibiotics: molecular properties of R-factors. *Ann. Rev. Microbiol.* 27:437-470.
- Juni, E. 1972. Interspecies transformation of Acinetobacteria, genetic evidence for a ubiquitous genus. *J. Bacteriol.* 112:917-931.
- Kelch, W. J. and J. S. Lee. 1978. Antibiotic resistance patterns of gram-negative bacteria isolated from environmental sources. *Appl. Environ. Microbiol.* 36:450-456.
- Kobori, H., C. W. Sullivan, and S. Shizuya. 1984. Bacterial plasmids in Antarctic natural microbial assemblages. *Appl. Environ. Microbiol.* 48:515-518.
- López-Torres, A. J., T. C. Hazen, and G. A. Toranzos.

1987. Distribution and in situ survival and activity of *Klebsiella pneumoniae* and *Escherichia coli* in a tropical rain forest watershed. *Curr. Microbiol.* 15: 213–218.
- Mach, P. A. and D. J. Grimes. 1982. R-plasmid transfer in a wastewater treatment plant. *Appl. Environ. Microbiol.* 44:1395–1403.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, New York. 783 pp.
- Niemi, M., M. Sihakov, and S. Niemela. 1983. Antibiotic resistance among different species of fecal coliforms isolated from water samples. *Appl. Environ. Microbiol.* 45:79–83.
- Oluwande, P. A., K. C. Sridhar, A. O. Bammeko, and A. O. Okubadejo. 1983. Pollution level in some Nigerian rivers. *Water Res.* 17:957–963.
- Rivera, S., T. C. Hazen, and G. A. Toranzos. 1988. Isolation of fecal coliforms from pristine sites in a tropical rain forest. *Appl. Environ. Microbiol.* 54: 513–517.
- Santiago-Mercado, J. and T. C. Hazen. 1987. Comparison of four membrane filtration and MPN methods for fecal coliform enumeration in tropical waters. *Appl. Environ. Microbiol.* 53:2922–2928.
- Santo Domingo, J. W., F. A. Fuentes, and T. C. Hazen. 1989. Survival and activity of *Streptococcus faecalis* and *Escherichia coli* in petroleum-contaminated waters. *Environ. Pollut.* 56:263–281.
- Shaw, D. R. and V. J. Cabelli. 1980. R-plasmid transfer frequencies from environmental isolates of *Escherichia coli* to laboratory and fecal strains. *Appl. Environ. Microbiol.* 40:756–764.
- Smith, H. W. 1970. Incidence in river water of *Escherichia coli* containing R factors. *Nature* 228:1286–1288.
- Smith, H. W., B. D. Danner, and R. Deich. 1981. Genetic transformation. *Ann. Rev. Biochem.* 50:41–68.