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Phenotypic and Genotypic Comparison of *Escherichia coli* from Pristine Tropical Waters

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Nine fecal-coliform-positive strains were isolated from pristine sites in a tropical rain forest. These sites included nonpolluted rivers and water from bromeliads (epiphytes) which were 30 ft (ca. 910 cm) above the ground. Phenotypically, all of these isolates were identified as *Escherichia coli*. Their DNA was isolated and purified, and the base composition (G+C content) was determined and compared with that of *E. coli* B (ATCC 11303). The DNA from the environmental isolates was also hybridized to radiolabeled DNA from *E. coli* B. Eight strains had a DNA base composition similar to that of *E. coli* B and gave more than 75% homology with *E. coli* B. One strain had a different DNA base composition and a relatively low percentage of homology with the reference strain. The finding of *E. coli* in pristine tropical waters suggests that this bacterium could be a natural inhabitant in these environments and is not a reliable indicator of recent human fecal contamination in tropical waters. The indicators that are currently used in the tropics to test the biological quality of water should be reevaluated.

The waters of Puerto Rico are believed to be highly contaminated with human waste; the number of gastroenteritis cases reported annually has grown from 2,922 in 1971 to 56,000 in 1986. The disease seemed to spread even faster in 1987, possibly exceeding 70,000 cases. The standard procedures for the detection and enumeration of coliforms, i.e., most-probable-number and membrane filter techniques, are used by both temperate- and tropical-zone nations to assess the quality of water (1). However, there is strong evidence that these fecal-coliform assays are inadequate for the tropics (3, 21) and especially Puerto Rico (5, 12, 22). Several studies in the Mameyes River rain forest watershed of Puerto Rico showed that the fecal indicator *Escherichia coli* could survive, remain physiologically active, and grow at rates dependent on nutrient levels (12). This greatly contrasts with the situation encountered in temperate environments, where it is well documented that *E. coli* does not survive well in fresh waters (27, 28). In fact, this characteristic of nonsurvival in extraenteral environments is one of the major underlying assumptions for the use of *E. coli* as an indicator of fecal contamination in water (3). In marine environments it has been found that *E. coli* is able to survive for only 9 h after exposure to pristine tropical marine waters; thus, densities of fecal-coliform bacteria are surprisingly low at sites very close to a source of human sewage (23). However, it has also been observed that when nearshore coastal waters receive heavy organic loading, this trend is reversed (i.e., *E. coli* survives for extended periods) (23, 31).

In Puerto Rico, less than 30% of fecal-coliform-positive isolates on selective media are identified as *E. coli* by biochemical tests. Thus, the finding of *E. coli* in extraenteral tropical freshwater environments strongly suggests that this species could be a natural inhabitant of tropical aquatic environments or that the species found is one which phenotypically resembles *E. coli*. It is very important, in terms of the accuracy of the coliform assays currently used in the tropics, that the identity of these *E. coli*-like isolates be confirmed by a genetic homology study. In this study, the environmental isolates were compared with a reference strain in terms of overall genetic homology.

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MATERIALS AND METHODS

Bacterial procedures. The reference strain used in this study was *E. coli* B (ATCC 11303) obtained directly from the American Type Culture Collection, Rockville, Md. The environmental strains (test strains) were isolated from water samples collected from different sources. Strain FC5 was isolated from a small stream 0.5 km from Dos Bocas Lake in Arecibo. This stream did not receive effluents from any sewage treatment plant; the area around the stream was not populated or urbanized, and there were no visible signs of domestic or wild animals. Strain FCW was isolated from site 1, a pristine site at the Mameyes River watershed, as described by Carrillo et al. (5) and López-Torres et al. (22). Strains BI#1, CI, CK, BB, AL, EM, and CJ were isolated from water accumulated in leaf axils of *Guzmania berteroniana*, one of the most abundant bromeliads (epiphytes) growing in the rain forest. A *Klebsiella pneumoniae* strain isolated from the Virgin Islands of the United States during an experimental cruise was used as a negative control in the hybridization experiments.

The fecal-coliform membrane filtration technique was used to isolate the environmental strains (1). Samples of water were filtered through a 0.7-μm-pore-size, HC-type membrane filter (Millipore Corp., Bedford, Mass.), and the filter was placed on m-FC medium (Difco Laboratories, Detroit, Mich.) and incubated for 24 h at 44.5°C. Blue colonies growing on m-FC were streaked on MacConkey medium (Difco) and incubated overnight at 35°C. Random isolates derived from pink colonies on MacConkey plates were Gram stained and identified biochemically with API 20E strips (Analytab Products, Plainview, N.Y.). All environmental isolates picked from m-FC and MacConkey plates

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were 99% similar to the API 20E profile for *E. coli*. Both reference and test strains were maintained on tryptic soy agar (Becton and Dickinson Co., Cockeysville, Md.). When needed, cells were grown at 35°C in tryptic soy broth until an exponential phase of growth was achieved.

DNA melting point. The procedure used for DNA isolation is a variation of the Marmur (25) technique as given by Johnson (15, 18). The extracted DNA was stored in a freezer at −20°C. To determine the amount of nucleic acid, 1:100 dilutions in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were read at wavelengths of 260 and 280 nm. A preparation was considered pure when it had an optical density at 260 nm/optical density at 280 nm ratio of 1:8 (24); if it had a ratio less than the value given, the preparation was assumed to be contaminated with protein and therefore was further purified.

A mini-Sub DNA agarose electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) was used to analyze the physical state of the DNA and its possible RNA contamination. The minigels were stained with ethidium bromide, and the DNA bands in the agarose gels were visualized by UV illumination of the gel.

The thermal denaturation method (17) was used for measuring DNA base composition. Samples (5 ml) of DNA at 50 μg/ml were prepared; 0.5× SSC was used to dilute the stock DNA preparations. A 10-ml volume of the standard DNA (i.e., the DNA of *E. coli* B, which has a G+C content of 51.7 mol% and a thermal melting pint [T_m] of 90.5°C in SSC) was prepared and used in each instrument run as a reference standard. The melting profiles were determined with a DU-8 UV spectrophotometer (Beckman Instruments, Inc., Palo Alto, Calif.). The moles percent G+C for the test DNA was determined by using the Marmur and Doty (26) equation.

DNA homology. In this study, [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) was used as a DNA precursor to label the reference DNA. To 500 ml of tryptic soy broth was added 0.5 mCi of [^{3}H]thymidine (specific activity, 76.1 Ci/mmol), which was then inoculated with 10 ml of an overnight culture of *E. coli* B. The DNA from the labeled cells was isolated by the method described above, and the final DNA preparation was dissolved in 0.1× SSC and fragmented by sonication. Labeled DNA concentrations were in the range of 100 to 130 μg/ml, and specific activities were 1,000 cpm/μg. The procedure of Maniatis et al. (24) for binding DNA to nitrocellulose was used to immobilize both reference and test DNA. The denatured DNA was filtered through a hemagglutination 96-well filtration plate (Millipore) consisting of a nitrocellulose membrane filter sealed to the bottom of a standard 96-well plate. After immobilization, the filter was air dried for 1 h and then washed twice with 50 ml of 6× SSC at room temperature. The filter was allowed to air dry for an hour and was then baked overnight at 60°C in a conventional oven. The plate was stored at room temperature over $CaSO_4$ in a desiccator. The remaining DNA-binding sites on the nitrocellulose filter were covered by prehybridizing a hemagglutination 96-well filtration plate containing both immobilized, unlabeled reference DNA and test DNA with Denhardt (6) solution (1×) at 60°C (25°C below the T_m) for 4 h.

After the preincubation period, the 96-well plate was filled with 100 μl of fresh prehybridization solution. Labeled *E. coli* B DNA (i.e., reference DNA) was denatured by being heated at 100°C for 10 min and then being chilled immediately in ice. Labeled reference DNA (10 μl; 100 μg/ml; specific activity, 1,000 cpm/μg) was added to each well containing the prehybridization solution; the plate was then incubated for 15 h at 60°C. At the end of the hybridization period, the plate containing the membrane was washed with 2 300-ml volumes of 2× SSC (prewarmed at 60°C) for 5 min each. In an alternative procedure, the membrane was washed with 2 300-ml volumes of 2× SSC–0.1% sodium dodecyl sulfate followed by 1 300-ml volume of 1× SSC–0.01% sodium dodecyl sulfate (all prewarmed at 60°C) for 10 min each. The latter procedure gave better results in terms of lower background counts. After being washed, individual membranes were removed from the wells in the filtration plate, air dried, and counted with a liquid scintillation counter (Beckman).

The percentage of homology was calculated by dividing the counts per minute bound by the heterologous DNA membranes (the membranes that contained immobilized, unlabeled test DNA hybridized to reference labeled DNA) by the counts per minute bound by homologous DNA membranes (the membranes that contained immobilized, unlabeled reference DNA hybridized to reference labeled DNA) and multiplying by 100.

RESULTS AND DISCUSSION

All of the environmental isolates were phenotypically identified as *E. coli* with >99% certainty; however, not all of the strains showed identical phenotypic profiles (Table 1). The presence of β-galactosidase activity (*o*-nitrophenyl-β-D-galactopyranoside test) has been used as a major criterion in the classification of members of the family *Enterobacteriaceae* (11); it was found that strain BI#1 did not produce this enzyme. Strains BB and AL produced the enzyme arginine dihydrolase but not ornithine decarboxylase; the latter enzyme was not produced by strain EM, either. These characteristics are valid criteria for the positive identification of the previously mentioned strains as *E. coli* because the genus *Escherichia* is made up of phenotypically variable strains; 11 to 25% of the strains are arginine dihydrolase positive, and 26 to 75% are ornithine decarboxylase positive (16). Although API 20E has been found to have the highest discriminatory potential and reproducibility in the family *Enterobacteriaceae* (32), it was designed for clinical isolates and may not be valid for environmental isolates, especially those from tropical environments.

E. coli DNA has a G+C content of 48 to 52 mol% (16), and most of the strains tested in this study had G+C contents that were within this range (Table 2). Strain AL had a T_m of 86°C and a G+C content of 49 mol%, which still places this strain within the species *E. coli*. Strain EM had a T_m of 85°C and a G+C content of 46.8 mol%, a value that differed by more than 2% from the moles percent G+C value for *E. coli*. Thus, strain EM is probably not *E. coli* but a different species. The T_m for the rest of the strains, including *E. coli* B, was 87°C, and the G+C content was 51.7 mol%.

A frequently used definition of a genetic species is "a group of strains whose DNA is 70% or more related at optimal reassociation conditions, 55% or more related at less than optimal reassociation conditions, and have 6% or less divergence in their related sequences" (4). However, various levels of relatedness ranging from 50 to 80% have been suggested as criteria for a species (30). Hermann et al. (13) stated that two strains belonging to a single species have DNA-DNA homologies exceeding 55%, while Döhler et al. (7) and Johnson et al. (19) have used a 60% criterion.

The results of DNA hybridization experiments are shown in Table 2. The strain included as a negative control, *K. pneumoniae*, showed a 34.0% ± 3.2% homology with *E. coli*

TABLE 1. Phenotypic characteristics of environmental isolates

Test	Response by strain:									
	Kp[a]	FC5	FCW	BI#1	CI	CK	BB	AL	EM	CJ
ONPG[b]	+	+	+	−	+	+	+	+	+	+
Arginine dihydrolase	−	−	−	−	−	−	+	+	−	−
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	−	+	+	+	+	+	−	−	−	+
Citrate	+	−	−	−	−	−	−	−	−	−
H_2S	−	−	−	−	−	−	−	−	−	−
Urease	+	−	−	−	−	−	−	−	−	−
Tryptophane deaminase	−	−	−	−	−	−	−	−	−	−
Indole	−	+	+	+	+	+	+	+	+	+
Voges-Proskauer	+	−	−	−	−	−	−	−	−	−
Gelatin	−	−	−	−	−	−	−	−	−	−
Nitrate reduction	−	+	+	+	ND[c]	ND	ND	ND	ND	ND
Glucose	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+
Inositol	+	−	−	−	−	−	−	−	−	−
Sorbitol	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	−	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+
Amygdalin	+	−	−	−	−	−	−	−	−	−
L-(+)-Arabinose	+	+	+	+	+	+	+	+	+	+

[a] Kp, *K. pneumoniae*.
[b] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside.
[c] ND, Not done.

B. This value is in complete agreement with that reported in *Bergey's Manual of Systematic Bacteriology* (16). The river isolate FCW showed a 74.0% ± 15.0% homology with *E. coli* B and a coefficient of variation of 17.0, while FC5 strain had a 78.2% ± 13.6% homology and a coefficient of variation of 10.9. The bromeliad isolate BI#1 had a 57.0% ± 3.0% homology and a mean coefficient of variation of 26.2. Considering that these strains showed the same G+C content as *E. coli* and had ≥60% homology with the reference strain, it can be concluded that FCW, FC5, and BI#1 belong to the same species as *E. coli*. BI#1 strain was also included in a DNA hybridization test developed by GENE-TRAK systems (Framingham, Mass.) in which a specific *Salmonella* sp. ^{32}P-radiolabeled DNA probe was hybridized to BI#1 DNA. The positive control in this experiment was DNA from *Salmonella typhimurium*. BI#1 had only 3% homology with the *Salmonella* probe, while the *E. coli* negative control had a very similar 2.8% homology. These results demonstrate that strain BI#1 does not belong to *Salmonella* sp.

K. pneumoniae and strains FCW, FC5, and BI#1 were tested in several DNA-DNA hybridization experiments. Initially, Denhardt solution was used as a blocking solution to avoid nonspecific binding. However, the use of Denhardt solution alone gave high background counts per minute. A prehybridization solution containing sodium dodecyl sulfate and salmon sperm DNA was used instead, and the membranes were washed at higher-stringency conditions of low salt concentration and higher washing-buffer volumes for a longer washing time. The latter procedure lowered the background counts significantly; this could be seen in the comparison of counts obtained in the nonspecific binding control experiment with counts obtained in the rest of the experiments.

The bromeliad isolates CK, EM, CI, BB, AL, and CJ showed DNA homology values that ranged from 54 to 75% (Table 2). The lowest values corresponded to strains CK (54%), BI#1 (57% ± 3.0%), and CJ (58%), and the highest values corresponded to strains CL (63%), AL (68%), and BB (75%). They also showed a similar *E. coli* B G+C content; therefore, these strains were considered *E. coli*. Strain EM, however, differed significantly in G+C content from *E. coli* B and did not show a high degree of homology with this strain (55%). Both results suggest that this strain is not *E. coli*.

Finding *E. coli* in pristine environments is extremely unusual because this bacterium inhabits the intestine of warm-blooded animals and its presence is expected only in environments that have been exposed to recent fecal contamination. Furthermore, it seems that this bacterium is capable of surviving indefinitely in a tropical environment (5, 12, 22, 31). This suggests that *E. coli* could be a natural inhabitant in these environments and that it may be part of a previously established community.

Total and fecal coliforms in Puerto Rico may not be reliable indicators of recent fecal contamination for various

TABLE 2. DNA base compositions and homology of reference and test strains

Strain	T_m (°C)	Mol% G+C	% Homology[a]
K. pneumoniae	ND[b]	ND	34.0 ± 3.2[c]
E. coli B	87	51.7	100.0 ± 12.6
FC5	87	51.7	78.2 ± 13.6
FCW	87	51.7	74.0 ± 15.0
BI#1	87	51.7	57.0 ± 3.0
CJ	87	51.7	58
EM	85	46.8	55
AL	86	49.0	68
BB	87	51.7	75
CK	87	51.7	54
CI	87	51.7	63

[a] Calculated as follows: (mean counts per minute bound by the heterologous DNA/mean counts per minute bound by the homologous DNA) × 100.
[b] ND, Not done.
[c] Mean ± 1 standard error.

reasons. It is assumed that *E. coli* is not able to survive or replicate outside the intestinal environment, and therefore its presence in water is presumed to indicate recent fecal contamination. Diffusion chamber studies with *E. coli* have demonstrated that this bacterium is capable of replicating and surviving indefinitely in tropical fresh waters (5). The currently used media for the isolation of fecal coliforms is not sensitive enough, because a large number of false-positives and false-negatives are detected when Puerto Rican waters are analyzed (12). Total and fecal coliforms have been isolated from water in bromeliads 30 ft (ca. 910 cm) above the ground and from pristine mountain streams (12). This study has demonstrated that these bacteria are in fact *E. coli*. All of this evidence suggests that the standard procedures currently used to test the biological quality of water in the tropics should be reevaluated.

The reliability of total and fecal coliforms as indicators in the tropics has been seriously questioned. These standard procedures may work well in temperate countries, but the tropics are different environments, in which the warm temperatures are relatively constant throughout the year. An alternative procedure for the assessment of water quality in the tropics could be the development of DNA probes to directly detect the pathogens present in water. The advantages of using DNA probes are as follows: they are highly specific and sensitive, no culturing medium (enrichment or selective) is needed, a given species or strain can be detected in a mixed culture, and a large number of samples can be screened easily. One disadvantage could be the cross-binding of the probe with conserved DNA nucleotide sequences among members of the same family (for example, *Enterobacteriaceae*). DNA probes have been developed for the identification of *Salmonella* spp. in food (10), enterotoxigenic *E. coli* (2, 14, 29), *Legionella* spp. in culture (8), *Bacteroides* spp. (20), and *Pseudomonas fluorescens* (9); development of other probes should make direct detection a reality and indicator assays obsolete.

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