Heterogeneity of uidA gene in environmental *Escherichia coli* populations

Clarivel Lasalde, Roberto Rodriguez, Henry H. Smith and Gary A. Toranzos

ABSTRACT

Previous studies have shown that *Escherichia coli* can be isolated from non-polluted rivers and from bromeliad axilae in pristine areas of tropical rain forests. Finding *E. coli* in pristine environments is unusual because this bacterium is thought to only survive in the gut of warm-blooded animals and thus its presence should indicate recent fecal contamination. The aims of this study were 1) to determine if *E. coli* is part of the native soil microbiota in tropical rain forests and 2) to determine if genetic heterogeneity exists among *E. coli* populations. High concentrations of total coliforms (10^4–10^5 cells per 10 g of soil dry weight) and low concentrations of thermotolerant coliforms (10^1–10^2 cells per 10 g dry soil, the majority of these were found to be *E. coli*) were detected. PCR using uidA-specific primers was done on DNA purified from *E. coli* isolates and the resulting amplicons analysed by denaturing-gradient gel electrophoresis (DGGE). Out of several hundred isolates, mixtures of nine different amplicons were consistently observed. The different patterns of DGGE observed indicate that the *E. coli* populations in these pristine soils are genetically heterogeneous. Fecal and environmental *E. coli* isolates were also analysed by pulsed-field gel electrophoresis (PFGE) which showed high DNA sequence variation among the *E. coli* isolates. Because of these differences in the genomes, PFGE did not allow grouping of environmental versus human isolates of *E. coli* when compared side to side. The apparent genetic polymorphisms, as a result of genetic heterogeneity, observed in isolates from the same pristine site indicate that source tracking may be difficult to carry out using *E. coli* as the target organism.

Key words | Environmental E. coli, tropical ecology, microbial diversity

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INTRODUCTION

Public and environmental health protection requires safe drinking and bathing waters, which must be free of pathogenic bacteria. Among the pathogens transmitted by water enteric pathogens are the ones most frequently encountered. Consequently, sources of fecal pollution in water and its microbiological quality must be strictly regulated (Rompre *et al.* 2002). Indicator organisms are globally used as a warning of possible contamination and as an index of water quality deterioration (Toranzos & McFeters 1997). The use of the coliform group, and more specifically the thermotolerant coliform, *Escherichia coli*, as an indicator of microbiological water quality is based on the assumption that *E. coli* cannot survive for extended periods of time extraenterally and can only originate from fecal sources. However, previous studies have shown that *E. coli* can be isolated from non-polluted rivers and from bromeliad axilae in pristine areas of tropical rain forests in Puerto Rico (Carrillo *et al.* 1985; López-Torres *et al.* 1987; Bermudez & Hazen 1988; Rivera *et al.* 1988) and Hawaii (Hardina & Fujioka 1991).

These findings go against the accepted idea that this bacterium cannot survive in the environment and can only

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replicate and survive in the gut of warm-blooded animals. Thus, the indicator bacteria could be present without any source of fecal contamination and could be a natural inhabitant in tropical waters (Bermudez & Hazen 1988) and soils (Hardina & Fujioka 1991). Although it can be argued that birds and mammals could be sources of *E. coli* in rain forest samples, low populations of these animals in El Yunque tropical rain forest and the continuous presence of *E. coli* in every single sample at different times of the day and during different days of the month and year refutes this hypothesis (Rivera *et al.* 1988).

Sources of thermotolerant coliform bacteria include runoff from feedlots and manure-amended agricultural land, wildlife, inadequate septic systems, urban runoff and sewage discharges. The ability to distinguish between human and animal sources of fecal contamination is an important assessment tool, both for evaluating possible health risks and for developing effective control strategies (Johnson *et al.* 2002). Although there have been numerous attempts to discriminate between human and non-human sources of fecal contamination in a water body, these attempts have been generally unsuccessful (Buchan *et al.* 2001). Recently, a DNA fingerprint technique called pulse-field gel electrophoresis (PFGE) was described as promising for discriminating fecal bacteria from humans and animals (Kariuki *et al.* 2001). The Centers for Disease Control and Prevention (CDC) has been using this method to identify and trace pathogenic *E. coli* (including O157:H7) isolates from disease outbreaks associated with food and water (Nakatsu *et al.* 2002). Although this technique works well with individual isolates such as pathogens, it is not truly straightforward when dealing with complex environmental samples. In this study, we used PFGE to try to differentiate *E. coli* of fecal origin from environmental *E. coli* isolates.

The uidA gene, which encodes for β-D-glucuronidase, has been used for detecting *E. coli* in previous studies (Bej *et al.* 1991a; Martins *et al.* 1993; Tsai *et al.* 1993; McDaniels *et al.* 1996; Iqbal *et al.* 1997; Lasalde *et al.* 2003). Although, the uidA and uidR genes are present in *E. coli* and *Shigella* spp. the activity of the enzyme is limited to *E. coli*. However, some *E. coli* fecal isolates have been shown to be negative for this activity, although the genes for the enzyme are present in these isolates (Martins *et al.* 1993; McDaniels *et al.* 1996; Monday *et al.* 2001). Studies show that many MUG-negative *E. coli* strains, including the pathogenic serotype O157:H7, were detected after PCR amplification of the uidA gene (Bej *et al.* 1991*a*,*b*; Martins *et al.* 1993; Iqbal *et al.* 1997; Monday *et al.* 2001; Rompre *et al.* 2002).

The aims of this study were to determine if *E. coli* populations are indeed members of the resident microbiota of tropical pristine soils and to determine any genetic heterogeneity on the uidA gene in soil isolates. Most-probable-number (MPN) analysis were performed to monitor the population for a period of 8 months (September 2002 to April 2003) and the genetic heterogeneity was determined using uidA PCR/DGGE (denaturing-gradient gel electrophoresis) analysis of *E. coli* soil isolates.

MATERIALS AND METHODS

Soil strain isolation

Soil samples collected from the upper part of El Yunque tropical rain forest at Luquillo, Puerto Rico, were placed in sterile plastic bags and kept at 4–7°C until processed within 24 h. The upper part of the tropical rain forest soil is a protected national forest and there are no signs of wild fauna. Two 3 m^2 transects (S1 and S2) at a distance of 5 m from a stream were sampled over an 8-month period. Replicate samples parallel to each other were taken at 0–5 cm and 6–11 cm depths (referred to as S1 and S2). Samples were also taken at the lower part of El Yunque (Mameyes River, referred to as S3), which is an urbanized area and receives domestic and sewage effluents. All samples were analysed separately for the presence of total coliforms, thermotolerant coliforms and *E. coli* over time. Temperature, pH and water content of soil were measured at each sampling site. Random isolates were confirmed as *E. coli* using the API 20E (BioMerieux, Inc., France) identification system.

Water and fecal strain isolation

E. coli isolates were collected from seven tributary streams at El Yunque tropical rain forest. Surface water samples were collected from seven different streams in sterile bottles and keep between 4 and 7°C until processed, within 24 hours. *E. coli* was isolated from these samples using

standard membrane filtration procedure on mFC agar (Difco Laboratories, Michigan) and incubated at 44.5°C for 18–24 hours. All dark blue colonies on mFC were subcultured onto eosin methylene blue agar (EMB, Difco Laboratories, Michigan) and grown in MUG-containing media to test for uidA activity. Fecal *E. coli* strains were isolated from humans, dogs, cats and other warm-blooded animals using a rectal swab and sterile 0.85% saline solution followed by streak isolation onto EMB plates. Dark green to black colonies accompanied by a metallic green sheen across the surface of the media were again subcultured onto fresh EMB plates. Random colonies were confirmed as *E. coli* isolates using API 20E.

Total and thermotolerant coliforms

Most-probable-number (MPN) and membrane filtration methods were used for soil sample analyses according to Standard Methods (1989). Ten (10) g of soil sample were diluted in 90 ml of sterile 0.85% saline solution and one drop of Tween 80 (Sigma Chemicals Company, Missouri) for MPN and membrane filtration analyses. MPN estimates were performed in lauryl tryptose broth (LTB, Difco Laboratories, Michigan) and EC medium with MUG (Difco Laboratories, Michigan).

DNA extraction from *E. coli* isolates

E. coli isolates were inoculated into sterile tryptic soy broth (TSB, Difco Laboratories, Michigan) and incubated at 37°C overnight. One ml was centrifuged for 5 minutes at 12,000 rpm and the pellet resuspended in 1 ml of TES sucrose (8% sucrose, 50 mM NaCl, 20 mM Tris-HCl pH 8.0, and 1 mM EDTA pH 8.0). Lysozyme (Sigma Chemicals Company, Missouri) was added to a final concentration of 1 mg ml^{-1} to each sample and incubated at room temperature for 10 minutes; 800 μl of phenol:chloroform:alcohol isoamyl (25:24:1) was added and centrifuged at 14,000 rpm for 10 minutes at room temperature. The DNA was purified with two phenol:chloroform:alcohol isoamyl extractions followed by chloroform extraction. DNA was precipitated using two volumes of cold 95% ethanol and 0.1 volume of 3 M sodium acetate pH 5 followed by incubation at −20°C overnight. DNA pellets were washed twice with cold 70% ethanol, resuspended in 10 mM Tris-HCl (pH 8.0) and stored at −20°C.

PCR and DGGE

A 0.167-Kb fragment was amplified from the uidA gene of *E. coli* and *Shigella* spp. using primers UAL-1939 and UAR-2105 (Bej *et al.* 1991*a*). A GC clamp was attached to the 5′ end of primer UAL-1939, leading to UAL-1939GC (Farnleitner *et al.* 2000). The PCR reaction mixture was prepared as follows: 1x PCR buffer, 3 mM $MgCl_2$, a 200 μM of each deoxynucleoside triphosphate (Promega Corporation, Wisconsin), 0.5 μM of each primer, 1 U of Taq DNA polymerase (Promega Corporation, Wisconsin) and DNA templates in a final volume of 50 μl. All amplification reactions were done using a Rapid Cycler (Idaho Technology, Idaho) and were started at 80°C. Denaturation, annealing and extension temperatures were 94°, 50°, and 74° C for 35 s, 35 s and 90 s, respectively, as described by Farnleitner *et al.* (2000).

Denaturing gradient gel electrophoresis

PCR products were run on a parallel denaturing gel in order to separate slight nucleic acid sequence differences within the amplified uidA gene region. Parallel DGGE analysis was done as described by Muyzer *et al.* (1998), with the following modifications described by Farnleitner *et al.* (2000): a 10% polyacrylamide gel with a gradient from 48 to 68% denaturing agent (100% denaturant was 7 M urea and 40% deionized formamide). Gels were run at 100 V and 60°C for 16 h in a DCode Electrophoresis System (Bio-Rad Laboratories, California) and stained with Sybr Green (Sigma Chemical Company, Missouri) for 30 min, as described by the manufacturer.

Pulsed field gel electrophoresis

This procedure was designed by the CDC for its use in tracing *E. coli* O157:H7 outbreaks isolates (CDC 1996). Briefly, *E. coli* strains were subcultured on EMB and incubated at 37°C overnight. A single colony was transferred to TSA and incubated overnight at 37°C. A single colony was suspended to a range of 13–15% transmittance in 3 ml of TE buffer (100 mM Tris:100 mM EDTA, pH 8.0). Plugs were formed by

mixing 0.2 ml of cell suspension with 10 μl of 20 mg ml^{-1} Proteinase K (Sigma Chemicals Company, Missouri), and 0.2 ml of agarose (1.6% Pulsed Field Certified agarose (Bio-Rad Lab., California):1.0% SDS in 10 mM Tris:1 mM EDTA, pH8.0). This mixture was pipetted into plug moulds (Bio-Rad Lab., California). Solidified plugs were transferred to 1.5 ml of lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0: 1% Sarcosine: 0.5 mg ml^{-1} Proteinase K) for 16 to 20 h of incubation in a 55°C shaker water bath. The lysis buffer was removed, and the plugs washed six times with 15 ml of sterile TE buffer (10 mM Tris: 1 mM EDTA, pH8.0) for 20 min in a 50°C shaker water bath. Two 1-mm-thick slices of plugs were incubated with 100 μl restriction enzyme mixture containing 30 U of XbaI in 1x buffer (Promega Corporation, Wisconsin) and incubated at 37°C for 3 h. Electrophoresis was performed using a CHEF Mapper (Bio-Rad Laboratories, California) system in a 0.5x TBE running buffer. The electrophoretic conditions used were as follows: initial switch time, 2.16 s; final switch time, 54.17 s; run time, 20 h; angle, 120°; gradient, 6.0 V cm^{-1}; temperature, 14°C and a linear ramping factor. The resulting gels were stained for 30 min in 500 ml of sterile water containing 60 μl of ethidium bromide (10 mg ml^{-1}) and destained for 30 min in 1 litre of distilled water. Gel analysis was performed using the Diversity Database software (Bio-Rad Laboratories, California).

RESULTS

Bacterial concentrations and physicochemical variables

No seasonal fluctuations were observed in pH, water content or temperature as shown in Table 1. No significant differences (as determined by Anova analysis) were found in thermotolerant coliforms or physicochemical variables concentrations over time between samples. Table 1 shows the average thermotolerant coliform concentrations observed during the sampling periods.

uidA gene analysis by DGGE

Under the conditions described above, 205 *E. coli* soil isolates were screened by parallel DGGE. Each uidA PCR product led to a single band in the DGGE gel (Figure 1). Of the 205 soil isolates, eight different DGGE melting types (MT2–MT9) (i.e. bands reaching different positions in the gel) (Farnleitner *et al.* 2000) were identified in soils. All DGGE MTs were easily distinguished from each other.

Of the eight DGGE MTs obtained MT4 and MT6 were the most frequent among the 205 *E. coli* soil isolates analysed (Figure 3). Overall DGGE MTs abundance was as follows: MT1 was not detected in soil isolates, 15.1% were MT2, 5.9% were MT3, 24.4% were MT4, 2.0% were MT5, 23.9% were MT6, 10.7% were MT7, 15.6% were MT8, and 2.4% were MT9. Abundance of each DGGE MT varied with sampling date (Figure 4). Abundance differences throughout the sampling period and between the different soils sampled can be observed (Figures 4 and 5).

Fecal and environmental *E. coli* PFGE fingerprinting

Well-defined profiles of genomic DNA under XbaI digestion were obtained from 86 different *E. coli* isolates (Figure 2). Fragment sizes were between 30 Kbp and 600 Kbp. Cluster analysis of the different band patterns obtained places almost every *E. coli* isolate in a different cluster (data not shown). Our results indicate a different band pattern for almost all the *E. coli* isolates analysed demonstrating whole-genome heterogeneity in these populations (data not shown).

DISCUSSION

Thermotolerant coliforms and *E. coli* were detected in all soils sampled at every sampling date, as reported in previous studies (Rivera *et al.* 1988; Hardina & Fujioka 1991; Lasalde *et al.* 2003). As there are no known sources of fecal contamination at any of the sampling sites, this strongly demonstrates that this bacterium is a natural inhabitant of tropical rain forest soils. Previously, soil had been considered the most likely source for high concentrations of indicator bacteria naturally present in freshwater streams of Hawaii (Hardina & Fujioka 1991). It should be noted that *E. coli* is a facultative aerobe and can thus survive under aerobic as well as anaerobic conditions. Soils can be aerobic when dry or anaerobic when water-saturated, depending on

Table 1 | Total and thermotolerant coliform concentrations in soils, physicochemical variables measured, and DGGE melting types (MTs) obtained during the sampling period

Sampling date (month-year)	Soil sampled (S1, S2 & S3)	Total coliforms (CFU per 10 g soil)	Thermotolerant coliforms (CFU per 10 g soil)	Temp	Water content %	pH	DGGE MTs[1]
Sep-02[2]	S1	1.92E + 03	5.17E + 02	20	29.7	4.4	4,6,7,9
Oct-02[2]	S1	8.12E + 04	6.2E + 02	20	32	4.5	6,9
Oct-02[2]	S2	8.96E + 04	3.4E + 01	20	39.8	4.4	4,7,8
Jan-03[3]	S1	ND	1.7E + 02	20.3	32.2	4.4	2,3,4, 6,7,8
Jan-03[3]	S2	ND	2.6E + 01	20	46.2	4.3	2,4,6,7,8
Jan-03[3]	S3	ND	2.5E + 02	27	22	7.3	4
Feb-03[3]	S1	ND	3.1E + 02	20.3	34.2	4.9	2,3,4,5,6,7,8
Feb-03[3]	S2	ND	4.2E + 02	19.7	44.7	4.8	2,4,5,6,7,8
Feb-03[3]	S3	ND	4.6E + 02	28.2	11	6.6	4,5,7
Mar-03[2]	S1	2.75E + 03	6.5E + 01	22.9	34.8	4.7	2,3,4,7,8,9
Mar-03[2]	S2	1.24E + 01	0.0E + 00	23.6	39.3	5.1	ND
Mar-03[2]	S3	2.50E + 01	0.0E + 00	42.8	8	6.7	ND
Apr-03[2]	S1	5.56E + 02	1.2E + 02	27.2	31	5.1	4,6,7
Apr-03[2]	S2	3.80E + 04	1.6E + 02	28.6	43	4.9	6,7
Apr-03[2]	S3	1.01E + 04	1.6E + 03	42.4	11	6.7	2,4

ND, not determined
[1]PuidA melting types (MTs) of *E. coli* determined by DGGE
[2]Thermotolerant coliforms (MPN index per 10 g soil) determined using most-probable-number (MPN)
[3]Thermotolerant coliforms (CFU per 10 g soil) determined using membrane filtration (MF)

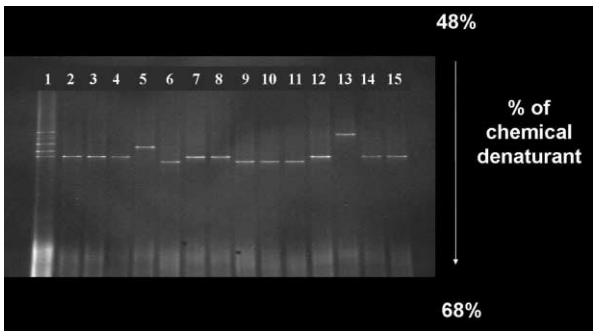

Figure 1 | Syber-Green staining of a parallel DGGE gel of *E. coli* soil isolates by PCR amplification of β-D-glucuronidase gene fragment (uidA) using primer pair UAL-1939GC and UAL-2105. Lane 1, MTs from different *E. coli* water isolates used in each DGGE as control. Lanes 2 to 15, *E. coli* isolates collected at a depth of 0 to 5 cm from soil 1 (S1) at time 3 (T3).

weather conditions, thus making the soil the most likely reservoir of indigenous *E. coli*.

DGGE analysis shows temporal and spatial heterogeneity in *E. coli* soil populations. Abundance differences in the eight different patterns were identified throughout the sampling period and between the different soils (Figures 4 and 5). Since DGGE efficiently separates the 208-bp uidA fragments, based on one to seven base-pair substitutions from each other (Farnleitner *et al.* 2000), it becomes obvious that the uidA fragments analysed have different base sequences and thus represent different clones. The obvious temporal changes in some melting types (MTs) suggest constant changes in some *E. coli* population over

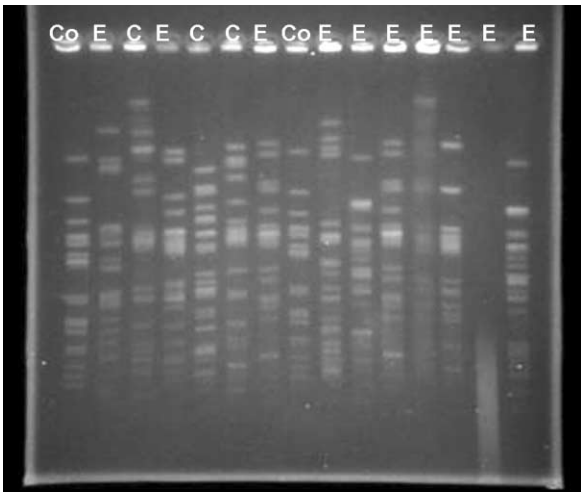

Figure 2 | *Xba*I restriction endonuclease fragments pattern of representative *Escherichia coli* isolates: *E. coli* K12 control strain (Co), environmental (E) and clinical (C) strains (12 isolates out of a total of 87 analysed are shown in this gel).

time, although there were MTs present in all samples. Natural bacterial communities have spatial and temporal heterogeneity and are constantly changing (Yannarell *et al.* 2003). Therefore, the *E. coli* soil populations should be constantly changing as a part of soil microbial communities. In addition, mutagenesis in ageing colonies (MAC) of *E. coli* occurs as a genetic strategy by-product for improving survival under stress, increasing genetic heterogeneity (Bjedov *et al.* 2003). In our study, only one gene was studied, and in fact only a portion of the gene coding for B-D-glucuronidase. The occurrence of different MTs may lead us to hypothesize that mutation rates may occur at a relatively high rate in the non-housekeeping genes.

Molecular typing of *E. coli* isolates recovered from different sources was done in order to see if they can be grouped into two categories, environmental and fecal.

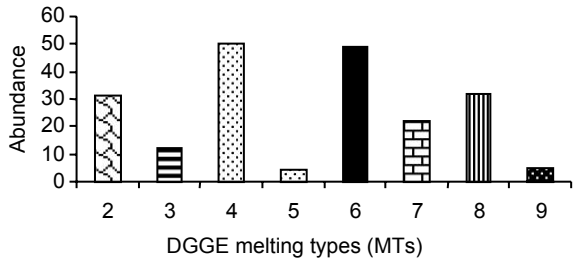

Figure 3 | Abundance of each DGGE melting type (MT) obtained by PCR amplification of uidA gene fragment. Of the nine MTs obtained MT4 and MT6 were the more frequent among the 205 *E. coli* soil isolates analysed.

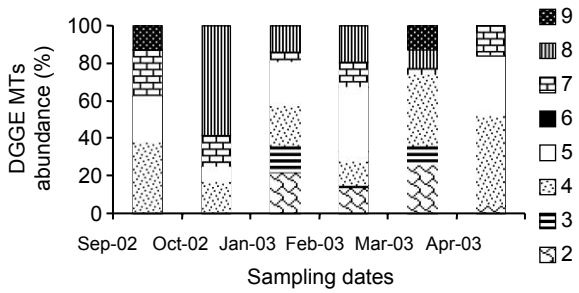

Figure 4 | Presence and percentage of DGGE MTs of uidA PCR products at six different sampling times: T1, September 2002; T2, October 2002; T3, January 2003; T4, February 2003; T5, March 2003; and T6, April 2003. DGGE MTs obtained vary between sampling times: T1 produced MT4, MT6, MT7 and MT9; T2 produced MT4, MT6, MT7 and MT8; T3 produced MT2, MT3, MT4, MT6, MT7 and MT8; T4 produced MT2 to MT8; T5 produced MT2, MT3, MT4, MT7, MT8 and MT9; and T6 produced MT2, MT4, MT6 and MT7.

Specifically, we tested pulsed field gel electrophoresis for identification of *E. coli* isolates source. Ideally, the PFGE patterns of isolates representing the environmental strains would be similar to each other and distinctly different from the unrelated fecal strains. Isolates are considered closely or possibly related if their PFGE patterns have the same number or almost the same number of bands (Tenover *et al.* 1995). In the present study, the DNA restriction patterns generated and resolved by PFGE failed to reveal any differences between the environmental and fecal *E. coli* isolates, but, perhaps most interestingly, neither the environmental nor the fecal strains shared band patterns amongst themselves to allow us to separate them into discrete groups. This heterogeneity may be due to random genetic events such as point mutations, insertions and deletions, thus making PFGE and perhaps DGGE too

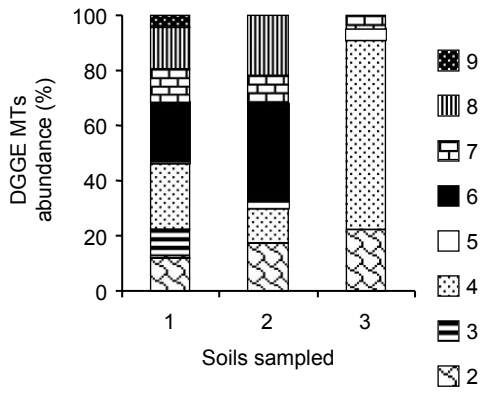

Figure 5 | Presence and percentage of DGGE MTs of uidA PCR products at the different soils sampled: S1 produced MT2 to MT9; S2 produced MT2, MT4, MT5, MT6, MT7 and MT8; and S3 produced MT2, MT4, MT5 and MT7.

sensitive to these mutation events and thus not very useful for source tracking purposes. Duplicate experiments using the same isolates displayed identical Xba I-PFGE profiles demonstrating the robustness of the method, and that genetic heterogeneity is a fact in *E. coli* populations.

Our data demonstrate that *E. coli* is in fact present in pristine soils in a tropical rain forest. Analysis of uidA gene using DGGE showed temporal and spatial genetic heterogeneity in these *E. coli* populations. Other studies had also demonstrated the presence of a high degree of genetic diversity among *E. coli* isolates using AFLP fingerprinting (Georgnaras *et al.* 2001; Dykes 2003). Molecular typing using PFGE did not allow grouping of *E. coli* isolates recovered from different sources into environmental or fecal groups. Therefore, spatial and temporal genetic heterogeneity of *E. coli* must be considered to effectively identify bacterial sources. In addition, because of the indigenous nature of *E. coli* considerations must be made about the importance of this bacterium as an indicator of recent fecal contamination, and the necessity of knowing the source of contamination if public health is to be protected.

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