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Proportion of β-D-Glucuronidase-Negative *Escherichia coli* in Human Fecal Samples

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Convenient assays and reports that almost all clinical isolates of *Escherichia coli* produce β-D-glucuronidase (GUR) have led to great interest in the use of the enzyme for the rapid detection of the bacterium in water, food, and environmental samples. In these materials, *E. coli* serves as an indicator of possible fecal contamination. Therefore, it was crucial to examine the proportion of GUR-negative *E. coli* in human fecal samples. The bacterium was isolated from 35 samples, and a mean of 34% and a median of 15% were found to be GUR negative in lauryl sulfate tryptose broth with 4-methylumbelliferyl-β-D-glucuronide. *E. coli* from three samples were temperature dependent for GUR production: very weakly positive at 37°C but strongly positive at 44.5°C. These results remind us of differences between fecal and clinical *E. coli* populations, of diversity in GUR regulation and expression in natural populations of *E. coli*, and of the need for caution in using GUR for the detection of fecal *E. coli*.

For much of this century, the coliform bacteria and especially *Escherichia coli* have been used as indicators of possible fecal contamination in water and food. Therefore, much effort has been expended on devising and improving methods for the detection, enumeration, and identification of these bacteria. Most of the methods in current use are based on the β-D-galactosidase activity of coliform bacteria and their ability to convert lactose to acid or gas (2, 3). Recently, however, another enzyme activity has shown great promise for the recognition and enumeration of *E. coli*. Kilian and Bülow examined clinical isolates of *E. coli* and found that about 97% of them produce β-D-glucuronidase (GUR), whereas most other coliform bacteria do not (19). This discovery, coupled with the development of convenient colorimetric and fluorometric tests for GUR, generated widespread interest in using the enzyme to enumerate *E. coli* quickly and easily in a variety of food, water, and environmental materials (10; P. A. Hartman, *in* A. Turano, ed., *Rapid Methods and Automation in Microbiology*, in press).

Because of the increasing use of GUR for detection of *E. coli*, we were surprised to find that most of the *E. coli* we isolated from laboratory rats failed to produce the enzyme. A preliminary survey of *E. coli* from a few human fecal samples gave similar results. Therefore, we examined the GUR-producing ability of *E. coli* from a larger number of human volunteers, and we present our findings in this report. To focus on the unexpectedly large proportion of GUR-negative *E. coli*, we report our results in terms of GUR-negative bacteria rather than, as presented by other authors, GUR-positive bacteria.

MATERIALS AND METHODS

Human fecal samples. To meet the combined requirements of voluntary participation, informed consent, confidentiality, and access to those involved to the results of their participation, we used the following protocol. After discussing the experiment with students, staff, and faculty of our department, we stocked each restroom with sterile cotton swabs in vials, along with instructions for collecting a small amount of fecal material on a swab and replacing the swab in the vial. Each vial was numbered, and results were posted as samples were analyzed. Therefore, volunteers could maintain their anonymity and yet learn what portion of their own fecal *E. coli* could produce GUR. Each donor was instructed to give only one sample. This protocol was reviewed and approved by the University of California Committee for the Protection of Human Subjects.

Bacterial isolation and characterization. Fecal samples were collected several times daily to assure bacterial viability. Swabs were soaked in 5 ml of 0.9% NaCl and agitated vigorously. Serial dilutions of the resulting suspensions were spread on MacConkey lactose agar (Difco Laboratories, Detroit, Mich.) and incubated for 24 h at 37°C. Then 10 to 20 lactose-positive colonies were transferred to Durham tubes containing 4-methylumbelliferyl-β-D-glucuronide (MUG) in lauryl sulfate tryptose (LST-MUG) broth (Difco). GUR activity was detected by the fluorescence of the broth when illuminated with a 6-W long-wavelength (366-nm) UV source. The negative controls were uninoculated broth and *Salmonella typhimurium* LT2; the positive control was *E. coli* ATCC 25922. Inoculated tubes were incubated in an incubator at 37°C or in a water bath at 44.5°C for up to 48 h, giving a profile or phenotype of growth, gas production, and GUR activity at the two temperatures for each isolate. Representative cultures of each phenotype from most fecal samples were then purified further on MacConkey lactose and Levine EMB agar and identified with the Enterotube II multiple-test kit (Roche Diagnostics, Div. Hoffmann-La Roche Inc., Nutley, N.J.). As a quality control measure, about half of the strains were retested with the API 20E system (Analytab Products, Plainview, N.Y.).

Statistical analysis. The chi-square test was used to compare the observed distribution of GUR-negative *E. coli* against models in which each fecal specimen was assumed to be a random sample of all existing fecal *E. coli*. This was done in each case by assuming that GUR-negative *E. coli* follow a Poisson distribution (29). The expected distribution was then divided into intervals which included a reasonable proportion of the expected samples. Thus, each expected distribution was divided into two to four sections, giving 1 to 3 degrees of freedom (df). Chi-square values were calculated from the differences between the observed and expected

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TABLE 1. Phenotypes and identities of lactose-fermenting bacteria isolated from human fecal samples

Phenotype						Species[a]					
37°C			44.5°C								
Growth	Gas production	GUR activity	Growth	Gas production	GUR activity	*Escherichia coli*	*Klebsiella pneumoniae*	*Klebsiella oxytoca*	*Klebsiella ozaenae*	*Enterobacter cloacae*	*Citrobacter freundii*
+	+	+	+	+	+	15 (10)	1 (1)		1 (1)		1 (1)
+	+	+	+	−	+	1					
+	+	+	−	−	−	1					
+	+	−	+	+	+	3 (2)[b]					
+	+	−	+	+	−	11 (8)	1 (1)	1 (1)			2 (2)
+	+	−	+	−	−		1 (1)			2[c]	1 (1)
+	+	−	−	−	−			(1)		3[d]	

[a] Shown are numbers of independent fecal samples from which bacteria of a given phenotype and species were isolated and identified. Parentheses indicate the number of isolates which were picked at random, tested, and confirmed by the API 20E system.
[b] Upon repeated testing, these isolates were found to be very weakly and variably GUR positive at 37°C.
[c] One was identified as *Serratia liquefaciens* by API 20E.
[d] One was identified as *Klebsiella oxytoca* by API 20E.

numbers of fecal samples in each section. To simplify calculations, we made the conservative assumption that each sample had yielded 10 *E. coli* isolates, even though most samples had yielded 20 or more. In summarizing our data and tabulating published data, we estimated the two-sided 95% confidence intervals from a graph of the confidence intervals of proportions following a binomial distribution (30).

RESULTS

We obtained 711 bacterial isolates from 39 fecal samples. Their phenotypes of growth, gas production from lactose, and GUR activity at 37 and 44.5°C are summarized in Table 1. Most fecal samples yielded bacteria of one or two phenotypes. Bacteria capable of growth, gas production, and GUR activity at both temperatures were found in 32 fecal samples. Bacteria from 16 of these 32 samples were examined further, and 15 were found to be *E. coli*. These 16 samples also contained *Klebsiella pneumoniae*, *Klebsiella ozaenae*, and *Citrobacter freundii*. Eleven fecal samples had *E. coli* which lacked GUR at either temperature. There was no fluorescence, even after they were adjusted to pH 8 to 9 with 2-amino-2-methyl-1-propanol. Three samples had *E. coli* which were strongly GUR positive at 44.5°C but negative after 24 h at 37°C. Upon repeated testing, these strains were found to be very weakly and variably GUR positive at 37°C; often they were negative at 24 h and weakly positive at 48 h. Among the lactose-fermenting members of the family *Enterobacteriaceae*, there were *K. pneumoniae* in a total of three samples, *Klebsiella oxytoca* in two, *K. ozaenae* in one, *Enterobacter cloacae* in three, and *C. freundii* in four.

The distribution of GUR-negative *E. coli* among fecal samples is shown in Fig. 1. Because we did not identify each of the 711 isolates, we made the following assumptions in constructing Fig. 1: (i) within a given fecal sample, the identity of an isolate could be assigned to all isolates which had identical phenotypes, (ii) all isolates with fully positive phenotypes (growth, gas production, and GUR activity) were *E. coli*, and (iii) in the five samples that contained GUR-negative coliforms but were not tested further, 60% of those bacteria were *E. coli*. The 60% estimate was calculated from the data in Table 1.

Among the 35 samples that yielded *E. coli*, 3 had entirely GUR-negative *E. coli*, 14 had exclusively GUR-positive isolates, and 18 had mixtures of negative and positive isolates (Fig. 1). The mean proportion of GUR-negative *E. coli* was 34% (95% confidence interval, 17 to 52%), and the median value was 15%. Or, expressed more conventionally in terms of GUR-positive *E. coli*, we found a mean of 66% positive, a value which is clearly inconsistent with a model in which 95% of *E. coli* are assumed to be GUR positive (chi square = 8.0, df = 1, $0.001 < P < 0.005$).

The data in Fig. 1 are also too spread out to be consistent with a model in which the fecal sample from each volunteer is a truly random sample of a universal pool of GUR-negative and -positive *E. coli*. Such a model would give a single peak at 34% GUR-negative *E. coli*, a sharp contrast to the spread of data shown in Fig. 1 (chi square = 12, df = 2, $P < 0.005$).

We measured the proportion of GUR-negative *E. coli* repeatedly in one subject and found it to vary from day to day. Samples taken every other day contained 9 of 17 (65%), 3 of 20 (15%), and 0 of 20 (0%) GUR-negative *E. coli*. This variation is too large to attribute to random sampling error (chi square = 18.5, df = 2, $P < 0.001$).

DISCUSSION

Fecal samples from healthy human subjects yielded an unexpectedly large proportion of GUR-negative *E. coli*. In

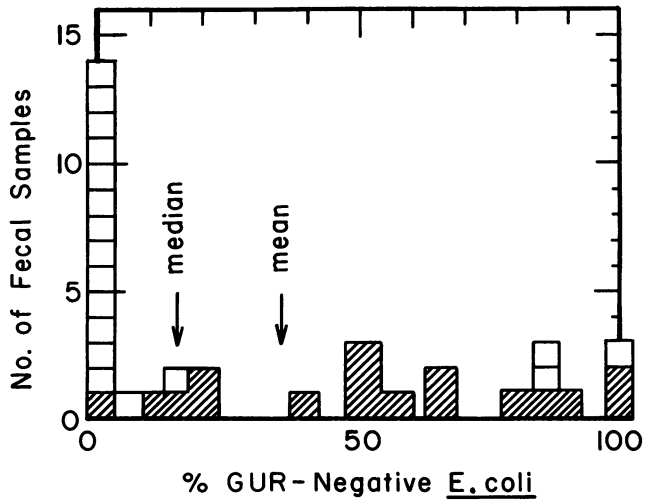

FIG. 1. Percentage of GUR-negative *E. coli* in human fecal samples. Each square represents one fecal sample. Cross-hatched squares represent samples from which representative bacteria were isolated and identified.

TABLE 2. Survey of published reports of the proportion of GUR-negative *E. coli*

Source of *E. coli*	Unit tested	GUR method[a]	GUR negative/total[b]	% GUR negative[c]	Reference
Urine	Isolated strain	Q_pNP	36/286	13 (10–17)	6
	Isolated strain	Q_pNP	22/354	6 (3–8)	9
	Isolated strain	Q_pNP	18/323	6 (3–8)	14
Clinical	Isolated strain	Q_pNP	19/169	11 (6–17)	7
	Isolated strain	MUG agar	14/206	7 (3–14)	17
	Isolated strain	Q_pNP	4/113	3 (1–9)	19
	Isolated strain	Q_pNP	28/609	5 (3–7)	21
	Isolated strain	MUG agar	3/72	4 (1–12)	34
Veterinary	Isolated strain	QMUG	2/269	5 (1–8)	16
Water supply	Isolated strain	QMUG	0/6	0 (0–50)	8
Surface water	Isolated colony	MUG agar	17/224	8 (5–12)	12
Seawater	Isolated colony	MUG agar	37/425	9 (7–12)	22
Foods	Food sample	MUG MPN	0/112 (10/112)[d]	0 (0–3) (9 [4–16])[d]	32
	Food sample	MUG MPN	0/117	0 (0–4)	24
Shellfish	MPN tube	MUG MPN	0/102	0 (0–4)	20
	MPN tube	MUG MPN	18/477	4 (2–6)	31
Sewage	Isolated colony	MUG agar	14/129	11 (6–19)	12
Pulp mill wastewater	Isolated colony	Q_pNP	2/30	7 (1–22)	26
Culture collection	Isolated strain	MUG MPN	4/110	4 (1–10)	11
Human feces	Isolated strain	Q_pNP	19/288	6.6 (4–10)	25
Human urine	Isolated strain	Q_pNP	0/197	0 (0–2)	25
Environmental	Isolated strain	Q_pNP	5/60	8.3 (3–18)	25
Rat intestinal tract	Isolated strain[e]	PTG	16/18	89 (65–98)	13

[a] Substrates included phenolphthalein glucoronide (PTG), *p*-nitrophenyl glucoronide (Q_pNP) and 4-methylumbelliferyl glucorinide (QMUG), in quick tests, MPN tubes (MUG MPN), or agar medium (MUG agar).

[b] Numerator is number of GUR-negative *E. coli*; denominator is usually the total number of *E. coli* strains. However, in those cases indicated, it is the total number of isolated *E. coli* colonies, *E. coli*-containing food samples, or *E. coli*-containing MPN tubes.

[c] Percent GUR-negative *E. coli* observed. Figures in parentheses are upper and lower limits of the 95% confidence interval.

[d] If *Escherichia hermanii* and *Escherichia vulneris* are included with *E. coli*.

[e] Strains were identified only as *Escherichia* spp.

this study, 34% of the fecal *E. coli* were GUR negative, a sharp contrast to the 3.5% reported in the widely quoted study of Kilian and Bülow (19). Our figure is much higher than any of those listed in Table 2 with the exception of the report of "*Escherichia* spp." from rat feces (13). The reasons for the discrepancy between the high incidence of GUR-negative bacteria in our study and the generally lower incidence in earlier reports are not clear. One reason could be differences between fecal and other sources of *E. coli*. The studies cited in Table 2 deal mainly with *E. coli* from clinical, food, and environmental sources. Most clinical isolates are from urinary tract infections, with bacteremias, diarrheal stools, and miscellaneous infections providing the rest of the *E. coli*. In contrast, the bacteria in this report came from the stools of healthy subjects. Perhaps the intestinal or fecal environments put less selective pressure on the genes involved in GUR production than do other environments. Such selective pressure need not act directly on GUR but may act on some nearby gene which is closely linked to those for GUR production. For example, an environment which selects for the *pil* genes, which determine production of type I fimbrial adhesions, might well select for retention of the nearby *uxu* genes, which control GUR production (27). These genetic loci are closely linked in *E. coli* K-12 (4). The data of Nastasi et al. support this hypothesis (25); GUR-negative *E. coli* comprised 6.6% of fecal and 0% of urinary isolates.

Other hypotheses include the possibility of a uniquely Californian fecal *E. coli* population which has an anomalously large proportion of GUR-negative variants. This seems unlikely, in light of serological, biotyping, and enzyme electrophoretic data suggesting a worldwide distribution of many *E. coli* genotypes (15). The hypothesis that our results may be attributable to the ethnic diversity of local students and staff members also seems unlikely. In the few cases where fecal donors identified themselves, there was no relationship between the incidence of GUR-negative *E. coli* and the ethnicity or presumptive lactose tolerance status of the host. Alternative hypotheses about the effects of diet, lifestyle, or hormonal status remain untested.

The distribution of GUR-negative bacteria raises the possibility that there are two kinds of people: those who carry GUR-negative bacteria and those who do not. The data in Fig. 1 are consistent with a model in which half the human population supports a fecal *E. coli* population with an average of only 5% GUR-negative *E. coli* and the other half carries an average of 85% GUR-negative *E. coli*. However, the large variation of GUR negatives in the subject who gave three fecal samples warns against such a simplistic picture. The downward drift in the proportion of GUR-negative *E. coli* in this subject is consistent with earlier reports in which the *E. coli* populations of individuals were studied for long periods of time. In studies reviewed by Hartl and Dykhuizen, there was a continual succession of *E. coli* strains, generating considerable drift in the proportions of several alleles (15). If GUR is a selectively neutral trait in intestinal *E. coli*, then its frequency would be expected to drift over time in any given subject.

Some of the apparent discrepancy between our data and earlier reports may simply reflect differences in how the results are reported. For example, in a very extensive study of foods, Moberg found GUR-positive *E. coli* in every food sample which actually contained *E. coli* (24). In other words, at least some of the *E. coli* in each of those food samples were GUR producers. In a similar way, a casual reading of reports of GUR-positive *E. coli* in MPN (most-probable-number) tubes might lead to misunderstanding. The MPN technique involves inoculating successive sets of MPN tubes

with serial dilutions of a test sample. Thus, positive tubes early in the series which were inoculated with more concentrated portions of test sample will have received far more bacteria than the later tubes which were given more dilute inocula. A test sample that contains a mixture of GUR-negative and GUR-positive *E. coli* will generate an MPN series in which all of the more concentrated tubes contain mixtures of positive and negatives. These tubes will be scored as GUR positive because the presence of positive bacteria masks the presence of negatives. Only those dilute MPN tubes which received exclusively GUR-negative *E. coli* will be scored as such. Thus, a count of MPN tubes can massively underestimate the incidence of GUR negatives. This effect was demonstrated elegantly in a study in which the same 30 selected seafood samples were analyzed both by MUG-containing MPN tubes and by plating on solid MUG-containing media (1). Only 1.1% (95% confidence interval of 0.5 to 2%) of the fecal coliform-containing MPN tubes were GUR negative. In contrast, 7% (95% confidence interval of 3 to 14%) of the isolated colonies of fecal coliforms were GUR negative.

Three subjects carried *E. coli* strains which produced GUR in a temperature-dependent fashion: strongly positive at 44.5°C but only weakly and variably positive at 37°C. These strains raise several interesting questions: First, do these strains have defects in the uptake of β-glucuronide substrates? Could they be similar to the cryptic strains of Kaspar et al., which lacked GUR activity in whole-cell assays but had immunologically detectable enzyme levels in cell extracts (18)? Or could they be similar to the *E. coli* strain which required several days of incubation before producing a measurable GUR activity (33)? Also, could the expression of GUR in some strains be a temperature-regulated trait, just as the expression of virulence genes seems to be in some bacteria (5, 23)? Finally, could GUR be produced as a heat shock protein or stress factor in these isolates (21, 35)?

Regardless of their implications about *E. coli* population structure and molecular genetics, our findings have clear significance for food, water, and sanitary microbiology. First, although tests for GUR are fast and simple, they may overlook up to a third of the *E. coli* of fecal origin. For example, food or water contaminated with excrement from the subjects whose results are depicted on the righthand edge of Fig. 1 would give false-negative GUR tests for *E. coli*. Second, in our survey of proven lactose-fermenting bacteria, the classic fecal coliform property of gas production from lactose at 44.5°C gave fewer false-negative tests for *E. coli* than did GUR production (1 of 30, or 3%, versus 14 of 31, or 45%, with 95% confidence intervals of 1 to 15, and 25 to 63%, respectively, on the basis of data in Table 1). Third, the significant proportion of GUR-negative *E. coli* in fecal samples from healthy subjects reminds us that although GUR may be a convenient tool for detecting some *E. coli*, it cannot be used for taxonomic purposes.

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