Multilocus Sequence Typing Reveals a Lack of Diversity among *Escherichia coli* O157:H7 Isolates That Are Distinct by Pulsed-Field Gel Electrophoresis

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Escherichia coli **O157:H7 is a major cause of foodborne illness in the United States. Pulsed-field gel electrophoresis (PFGE) is the molecular epidemiologic method mostly commonly used to identify food-borne outbreaks. Although PFGE is a powerful epidemiologic tool, it has disadvantages that make a DNA sequence-based approach potentially attractive. Multilocus sequence typing (MLST) analyzes the internal fragments of housekeeping genes to establish genetic relatedness between isolates. We sequenced selected portions of seven housekeeping genes and two membrane protein genes (*ompA* and *espA*) of 77 isolates that were diverse by PFGE to determine whether there was sufficient sequence variation to be useful as an epidemiologic tool. There was no DNA sequence diversity in the sequenced portions of the seven housekeeping genes and *espA*. For *ompA*, all but five isolates had sequence identical to that of the reference strains. *E. coli* O157:H7 has a striking lack of genetic diversity in the genes we explored, even among isolates that are clearly distinct by PFGE. Other approaches to identify improved molecular subtyping methods for *E. coli* 0157:H7 are needed.**

Escherichia coli O157:H7 was first recognized in 1982 in association with a food-borne outbreak and is now recognized as an important cause of food-borne illness in the United States, causing an estimated 74,000 infections a year (20, 26). This pathogen causes both bloody diarrhea and hemolytic uremic syndrome (HUS), a severe illness characterized by hemolytic anemia and acute renal failure (2). In recent years, there have been numerous large outbreaks of *E. coli* O157:H7-related bloody diarrhea and HUS (3, 4, 11). Most *E. coli* O157:H7 infections are caused by exposure to food or water that has bovine fecal contamination.

Pulsed-field gel electrophoresis (PFGE) is currently the most widely utilized molecular subtyping method for detecting outbreaks of *E. coli* O157:H7. In fact, PFGE has been found to identify outbreaks of *E. coli* O157:H7 that were not detected by traditional epidemiologic methods (1). However, DNA sequence-based methods offer several important potential advantages over PFGE, including shorter assay times and fully comparable and transferable data between laboratories (5, 16, 19, 21). Additionally, while PFGE is relatively simple and inexpensive, it is labor intensive, the interpretation of banding patterns can be subjective, and it does not easily handle large sample sets (13).

Multilocus sequence typing (MLST) is a DNA sequence-based molecular subtyping method that has been used success-fully for other bacteria, such as *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Salmonella*, for both evolutionary and epidemiologic studies (8, 16, 22, 29). Briefly, MLST examines the alleles of selected housekeeping genes by nucleotide sequencing a 500- to 600-bp segment of the gene. The sequence data are then analyzed to determine the genetic relatedness of the bacterial isolates. In this study, we performed MLST on a set of *E. coli* O157:H7 isolates that had been characterized epidemiologically and by PFGE to determine the potential utility of MLST for the molecular subtyping of this organism.

MATERIALS AND METHODS

***E. coli* O157:H7 isolates.** *E. coli* O157:H7 isolates were obtained from several sources for this study. Isolates were selected to include groups of strains from known outbreaks that were indistinguishable by PFGE, groups that were indistinguishable by PFGE but not associated with a known outbreak, and strains with a unique PFGE pattern that were not known to be associated with an outbreak.

The Public Health Infectious Disease Laboratory (PHIDL) obtained all *E. coli* O157:H7 strains isolated by the Allegheny County Health Department (ACHD) from 1999 to 2001 (*n* = 59). These strains were not associated with known outbreaks, with the exception of seven isolates from a single restaurant-associated outbreak in August and September 2001. A sample of isolates from the Minnesota Department of Health (MDH) was also included; these were outbreak-associated isolates (*n* = 14) and sporadic isolates (*n* = 4) from 1996 and 1997. ATCC strain EDL933 and the Sakai, Japan, strain RIMD 0509952 were used as reference strains (10, 23).

PFGE. PFGE analysis was performed according to the Centers for Disease Control and Prevention PulseNet protocol with minor variations (25, 28). Briefly, pure isolates were grown overnight on blood agar. Equal amounts of bacterial suspension, represented by an optical density at 610 nm of 1.3 in 1× TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]; Sigma, St. Louis, Mo.), 1% SeaKem Gold agarose (BioWhittaker, Rockland, Maine), and 1% sodium dodecyl sulfate (Sigma) were added to 0.5 mg of proteinase K per ml (Sigma) and mixed to form

TABLE 1. Forward and reverse sequences of primers, based on sequences found in GenBank or those of published or unpublished primers

Gene	Primer sequence[a]	Reaction parameters[b]	Amplicon size (bp)[c]	Reference or accession no.
arcA	F: 5′-GAAGACGAGTTGGTAACACG-3′	95°C for 1 min, 55°C for 2 min, 72°C for 3 min, 30 cycles	680	17
	R: 5′-CTTCCAGATCACCGCAGAAGC-3′			
aroE	F: 5′-AAGGTGCGAATGTGACGGTG-3′	95°C for 1 min, 57°C for 2 min, 72°C for 3 min, 28 cycles	620	17
	R: 5′-AACTGGTTCTACGTCAGGCA-3′			
dnaE	F: 5′-GAG/TATGTGTGAGCTGTTTGC-3′	94°C for 45 s, 45°C for 45 s, 72°C for 1 min, 30 cycles	550	P. Garg, personal communication
	R: 5′-CGA/GATA/CACCGCTTTCGCCG-3′			
mdh	F: 5′-CAACTGCCTTCAGGTTCAGAA-3′	94°C for 45 s, 50°C for 45 s, 72°C for 1 min, 30 cycles	580	AE005551
	R: 5′-GCGTTCTGGATGCGTTTGGT-3′			AP002564
gnd	F: 5′-GGCTTTAACTTCATCGGTAC-3′	94°C for 45 s, 50°C for 45 s, 72°C for 1 min 10 s, 30 cycles	590	AE005428
	R: 5′-TCGCCGTAGTTCAGATCCCA-3′			AP002559
gapA	F: 5′-GATTACATGGCATACATGCTG-3′	94°C for 45 s, 50°C for 45 s, 72°C for 1 min 10 s, 30 cycles	535	AE005401
	R: 5′-CAGACGAACGGTCAGGTCAAC-3′			AP002558
pgm	F: 5′-CCG/TTCG/CCAG/TAACCCGCC-3′	94°C for 45 s, 50°C for 45 s, 72°C for 1 min, 35 cycles	600	13
	R: 5′-TCA/GACA/GAACCATTTGAAA/G/TCC-3′			
espA	F: 5′-ATGGATACATCAAA/CTGG/CA/CAC-3′	94°C for 45 s, 50°C for 45 s, 72°C for 1 min, 35 cycles	579	AE005594
	R: 5′-TTATTTACCAAGGGATATT-3′			AP002566
ompA	F: 5′-AGACAGCTATCGCGATTGC-3′	94°C for 45 s, 50°C for 45 s, 72°C for 1 min, 30 cycles	691	AE005286
	R: 5′-GCTTTGTTGAAGTTGAACAC-3′			AP002554

[a] F, forward; R, reverse.
[b] All reactions had an initial denaturation at 94°C for 4 min and a final extension at 72°C for 4 min.
[c] The sequenced DNA strands were typically shorter than the original PCR amplicon.

plugs. The bacteria were lysed within the plugs with a cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8], 1% Sarcosine, 0.1 mg of proteinase K per ml [Sigma]) and incubated overnight at 37°C. The plugs were then washed four times with 1× TE buffer. Two-millimeter slices of plugs were incubated overnight with either *Xba*I or *Spe*I (New England Biolabs, Beverly, Mass.) at 37°C. The plugs were then loaded onto a 1% SeaKem Gold agarose gel. PFGE was performed with the CHEF III system (Bio-Rad, Hercules, Calif.) with the following run parameters: *Xba*I with a switch time of 3 to 40 s and a run time of 21 h and *Spe*I with switch time of 3 to 20 s and a run time of 21 h. All gels were run with the Centers for Disease Control and Prevention reference strain, G5244, of *E. coli* O157:H7. After the gel had been stained with ethidium bromide, the gel was captured with the Gel Doc 2000 and Multi-Analyst program (Bio-Rad). Dendrograms were created with Molecular Analyst (Bio-Rad) by using the Dice coefficient, unweighted pair group method with arithmetic means (UPGMA), and a position tolerance of 1.3%. Isolates were considered highly related with 0 or 1 band difference with both *Xba*I and *Spe*I.

MLST. Genomic DNA was isolated with Prepman Ultra according to the manufacturer's instructions (Applied Biosystems, Foster City, Calif.). Seven housekeeping genes were amplified from the genomic DNA by using recombinant *Taq* DNA polymerase (Gibco-Invitrogen, Gaithersburg, Md.); reaction parameters varied depending on the primer set (Table 1). The following genes (coding for the proteins in parentheses) were included: *arcA* (aerobic respiratory control protein) and *aroE* (shikimate dehydrogenase) with primers as described by Reid et al. (24), *dnaE* (DNA polymerase III, α subunit), *mdh* (malate dehydrogenase), *gnd* (6-phosphogluconate dehydrogenase), *gapA* (glyceraldehyde 3-phophate dehydrogenase), and *pgm* (phosphoglucomutase). Also sequenced were the membrane protein coding genes *espA* (*E. coli* secreting protein A) and *ompA* (outer membrane protein A).

The oligonucleotide primers were designed based on the published sequences of the genes found in GenBank (Table 1). PCR products were purified with Multiscreen PCR plates (Millipore, Bedford, Mass.). PCR products were sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Initial denaturation was for 4 min at 94°C, followed by 25 cycles of denaturation at 96°C (30 s), annealing at 50°C (5 s), and extension at 60°C (4 min), with a final extension at 72°C (1 min). The sequencing products were run on an Applied Biosystems 3700 DNA sequencer. Both the forward and reverse strands were sequenced with the PCR primer set (Table 1). Raw sequences were interpreted with Phred (a base-caller program) and Phrap (an assembly program) and verified with Consed (a Unix-based graphical editor) (6, 7, 9). All sequences were aligned and compared by using ClustalX, a graphical multiple alignment program (12). Sequence results were compared to the reference strains from the National Center for Biotechnology Information (NCBI) EDL933 (AE005174) and Sakai RIMD 0509952 (BA000007) by using ClustalX.

RESULTS

A total of 77 *E. coli* O157:H7 isolates were studied: 59 from Pennsylvania and 18 from Minnesota. The PFGE patterns of a selected group of these isolates, chosen to demonstrate the range of diversity of isolates that were sequenced, are shown in Fig. 1. The genetic relatedness of these isolates ranged from around 75 to 100% with *Xba*I and around 80 to 100% with *Spe*I (data not shown).
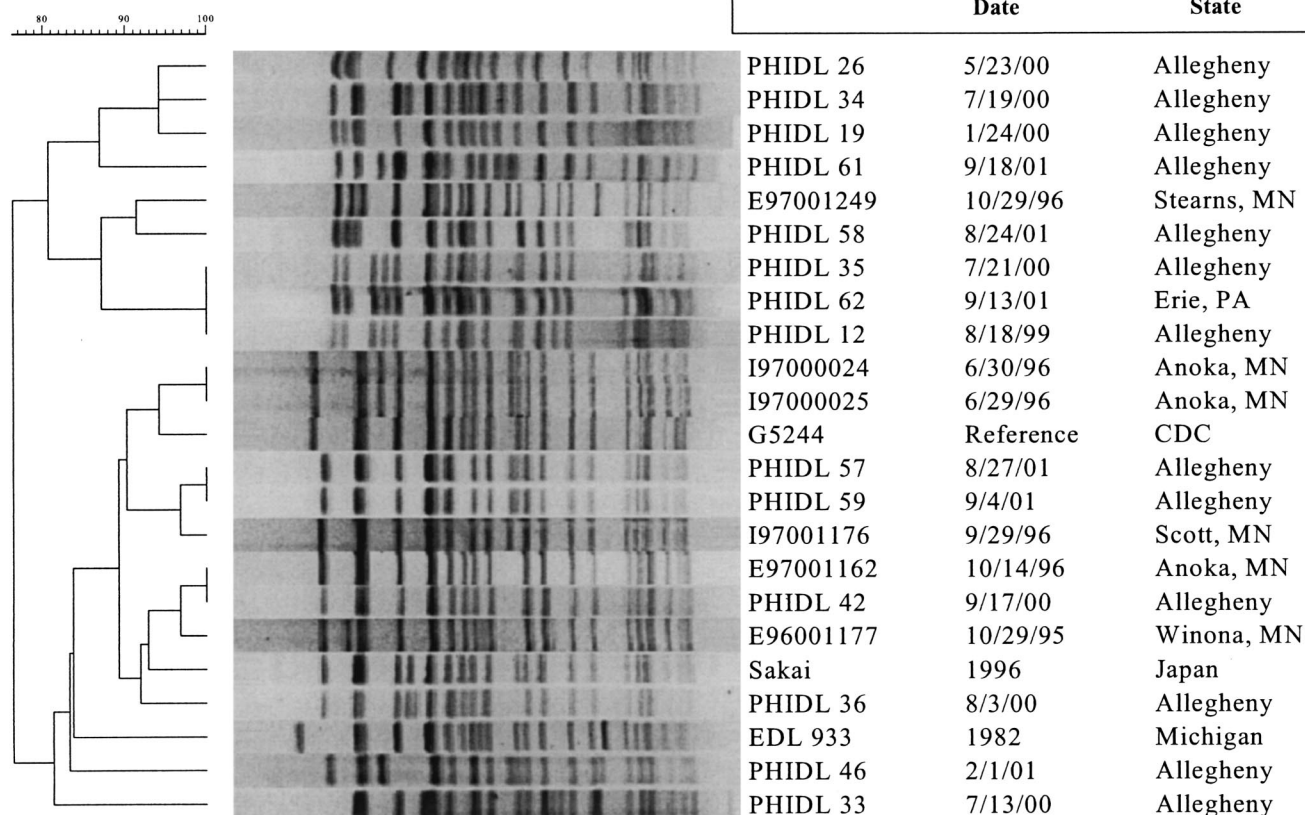
Isolate #	Isolation Date	County, State
PHIDL 26	5/23/00	Allegheny
PHIDL 34	7/19/00	Allegheny
PHIDL 19	1/24/00	Allegheny
PHIDL 61	9/18/01	Allegheny
E97001249	10/29/96	Stearns, MN
PHIDL 58	8/24/01	Allegheny
PHIDL 35	7/21/00	Allegheny
PHIDL 62	9/13/01	Erie, PA
PHIDL 12	8/18/99	Allegheny
I97000024	6/30/96	Anoka, MN
I97000025	6/29/96	Anoka, MN
G5244	Reference	CDC
PHIDL 57	8/27/01	Allegheny
PHIDL 59	9/4/01	Allegheny
I97001176	9/29/96	Scott, MN
E97001162	10/14/96	Anoka, MN
PHIDL 42	9/17/00	Allegheny
E96001177	10/29/95	Winona, MN
Sakai	1996	Japan
PHIDL 36	8/3/00	Allegheny
EDL 933	1982	Michigan
PHIDL 46	2/1/01	Allegheny
PHIDL 33	7/13/00	Allegheny

FIG. 1. PFGE analysis of selected strains from the ACHD and MDH restricted with *Xba*I. The reference strains include the following: G5244 (Centers for Disease Control and Prevention [CDC]), Sakai RIMD 0509952 (Japan), and EDL 933 (American Type Culture Collection). Isolates are designated by county and state, except for Allegheny County isolates, which are all from Pennsylvania. MN, Minnesota; PA, Pennsylvania.

Initially, housekeeping genes were targeted because they have successfully been used for other organisms (8, 22, 29). The seven selected housekeeping genes were chosen for their potential sequence diversity. Three of the genes, *aroE*, *arcA*, and *mdh*, have been used to determine the evolution of pathogenic *E. coli* (24). Two genes, *dnaE* and *pgm*, were chosen because they were found to be informative for *Salmonella* and *Vibrio cholerae* (16; Pallavi Garg, personal communication). The final two housekeeping genes, *gapA* and *gnd*, were chosen because they were transferred into the O157 genome at different evolutionary times. We hoped to find diversity as these genes reached G-C equilibrium with the new host (18). Finally, the two membrane proteins were chosen as being potential targets of the immune system and under possible pressure to mutate.

MLST analysis of the seven housekeeping genes demonstrated that the PHIDL and Minnesota strains had identical sequences at all seven loci. Similar to the housekeeping genes, there were no nucleotide differences in *espA*. All of the housekeeping genes and *espA* loci had identical sequences compared to the reference NCBI sequences EDL933 and Sakai RIMD 0509952. For *ompA*, all of the isolates had the same allele as the reference sequences in NCBI, except for five isolates that demonstrated two minor alleles. There was a single nucleotide polymorphism (SNP) (cytosine to thymidine) that was present

at base 301 in 4 PHIDL isolates (PHIDL no. 19, 26, 34, and 61). These isolates were clustered together by PFGE, but were not indistinguishable (Fig. 1). Additionally, PHIDL 61 did not remain in the cluster when digested with *Spe*I (data not shown). PHIDL 62 had an SNP further downstream in *ompA* at nucleotide 560 (guanine to adenine); but the other isolates in its *Xba*I PFGE cluster, PHIDL 12 and 35, did not have this nucleotide polymorphism and instead had the most common allele.

DISCUSSION

In this study, we found a striking lack of DNA sequence diversity for all seven housekeeping genes, with not a single difference in the approximately 311,000 nucleotides (over 4,000 nucleotides per isolate) that were sequenced. Additionally, two other genes which might have been expected to have a higher degree of diversity, *ompA* and *espA*, exhibited either minimal or no diversity, respectively. We had included these genes because we hypothesized that, with products exposed on the surface of the cell, they could be under immune pressure and therefore might exhibit a higher degree of genetic diversity than the housekeeping genes, as was seen in *Neisseria menigitidis* subgroup III (30).

In studies conducted with other bacterial species, strain-to-

strain variations in nucleotide sequence are commonly seen, even among strains within a single serotype (8, 19, 27, 29) and PFGE type (16) and/or associated with a common source. The observed sequence conservation could be interpreted as indicative of strong selection, as has been suggested for conserved genotypes of strains of *N. menigitidis* (19). An alternative interpretation—that the strains are clonal due to the organism's recent evolutionary appearance as a recently emerged human pathogen—is consistent with our sequence data. The contrast between the sequence conservation and presence of diversity as measured by PFGE that we observed could be explained if the PFGE pattern changes resulted from insertions and deletions of DNA that included a restriction site.

Three lines of evidence suggest that an important source of genetic diversity of *E. coli* O157:H7 is based on insertions and deletions of DNA sequences. First, octamer-based genome scanning has revealed distinct lineages of *E. coli* O157 strains. The polymorphic markers that distinguish the lines of descent have been shown to be the result of insertion and deletion of phages and prophages (14, 15). Second, the different banding patterns by PFGE have been shown to result from insertions and deletions containing the *Xba*I restriction sites, not SNPs (17). These deletion/insertion sites all were localized within O157-specific regions (O-islands) of the genome compared to the restriction sites *E. coli* O157:H7 has in common with *E. coli* K-12. Third, an analysis of the differences between the two published *E. coli* O157:H7 genomes indicated substantial differences attributable to insertions and deletions, because the total number of potential protein-encoding genes differs between the genomes by several dozen (10, 23). Additionally, Sakai RIMD 095520 has 1,632 O-island genes that are not found in *E. coli* K-12, while EDL933 has only 1,387 of these genes.

Sequence analysis has multiple advantages over fingerprinting-based methods, including shorter assay time, less subjectivity in interpretation of results, fully transferable data that are comparable among laboratories, and greater ease of automated computer analysis. Our study indicates that the genes we selected for analysis did not have sufficient variation to be useful as an epidemiological tool in *E. coli* O157:H7. Clearly, other approaches to identify informative regions of the genome will be required to develop improved methods for molecular subtyping of this important pathogen.

ACKNOWLEDGMENTS

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