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Phylogenetic Analysis of *Salmonella*, *Shigella*, and *Escherichia coli* Strains on the Basis of the *gyrB* Gene Sequence

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Phylogenetic analysis of about 200 strains of *Salmonella*, *Shigella*, and *Escherichia coli* was carried out using the nucleotide sequence of the gene for DNA gyrase B (*gyrB*), which was determined by directly sequencing PCR fragments. The results establish a new phylogenetic tree for the classification of *Salmonella*, *Shigella*, and *Escherichia coli* in which *Salmonella* forms a cluster separate from but closely related to *Shigella* and *E. coli*. In comparison with 16S rRNA analysis, the *gyrB* sequences indicated a greater evolutionary divergence for the bacteria. Thus, in screening for the presence of bacteria, the *gyrB* gene might be a useful tool for differentiating between closely related species of bacteria such as *Shigella* spp. and *E. coli*. At present, 16S rRNA sequence analysis is an accurate and rapid method for identifying most unknown bacteria to the genus level because the highly conserved 16S rRNA region is easy to amplify; however, analysis of the more variable *gyrB* sequence region can identify unknown bacteria to the species level. In summary, we have shown that *gyrB* sequence analysis is a useful alternative to 16S rRNA analysis for constructing the phylogenetic relationships of bacteria, in particular for the classification of closely related bacterial species.

Shigella and *Salmonella* are pathogens that cause gastroenteropathy in humans (22). Alimentary infections are mostly caused by *Salmonella*, which has a broad distribution throughout the natural world and a widespread occurrence in animals, especially in poultry and swine (10). *Shigella* spp. are, in fact, metabolically inactive biogroups of *Escherichia coli*, and some *E. coli* strains can cause diarrhea similar to that caused by *Shigella*. Brenner considered *Shigella* and *E. coli* to be a single species, based on DNA homology (4).

In general, the chromosomes of *Salmonella*, *Shigella*, and *E. coli* comprise a single circular DNA molecule consisting of about 4×10^6 bp, with a relative molecular mass of 4×10^9 and a total length of about 1.4 mm. *E. coli*, *Shigella*, and *Salmonella* all belong to the family *Enterobacteriaceae*.

There are over 2,000 *Salmonella* serotypes, based on antigenic differences associated with gastroenteritis and enteric (typhoid) fever in humans. The majority of these serotypes belong to a single *Salmonella* species, *Salmonella enterica* (23). *S. enterica* includes six subspecies (*Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *salamae*, *Salmonella enterica* subsp. *arizonae*, *Salmonella enterica* subsp. *diarizonae*, *Salmonella enterica* subsp. *houtenae*, and *Salmonella enterica* subspecies *indica*).

For *Shigella*, there are four species (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*) (3). Any of the four *Shigella* species can cause bacillary dysentery.

Finally, for *E. coli*, there are at least five pathotypes: enteroinvasive *E. coli*, enterotoxigenic *E. coli*, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (12, 24, 29, 35). Symptoms caused by EPEC resemble salmonellosis, those of enteroinvasive *E. coli* resemble *Shigella*, and those of enterotoxigenic *E. coli* resemble cholera.

Shigella and *E. coli* strains are often extremely difficult to separate biochemically because there are aerogenic (gas-producing) shigellae and lactose-negative, anaerogenic, nonmotile *E. coli* strains. *E. coli* strains can cause a shigella-like diarrhea, and *Shigella* species are regarded as metabolically inactive biogroups of *E. coli* (4). Therefore, it would be useful to classify these types of bacteria to aid the treatment of bacterial infections.

PCR has been used to determine the evolutionary relationships of bacteria by analyzing nucleotide sequences of various genes, including 16S/23S rRNA, housekeeping genes, and invasion genes (1, 2, 16, 19, 20, 26). In particular, 16S rRNA sequences have been widely used to construct bacterial phylogenetic relationships (6, 34) or to detect pathogenic bacteria (15). Bacterial analysis by 16S rRNA has become popular because these sections of RNA are conserved and easy to sequence. However, the classification of closely related species of bacteria, for example, *Shigella* spp. and *E. coli*, is difficult to achieve through 16S rRNA analysis (7).

As an alternative to 16S rRNA analysis, Yamamoto and Harayama (36, 37, 38) designed a set of PCR primers that allowed both the amplification of the *gyrB* gene, which encodes the subunit B protein of DNA gyrase (topoisomerase type II), from a large variety of bacteria and the rapid nucleotide sequencing of the amplified *gyrB* fragments. They then used the *gyrB* gene in the taxonomic classification of *Pseudomonas putida* and *Acetinobacter* strains. The sequences of *gyrB* genes imply that the rate of molecular evolution is higher than that determined by 16S rRNA sequences (9, 36, 37, 38). We thought that detailed analysis of bacterial phylogenetic relationships might be possible through the *gyrB* region, as this genetic region can classify bacteria that cannot be classified by their 16S rRNA regions. In particular, the *gyrB* gene region might be useful to analyze the phylogenetic relationship among

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TABLE 1. 16S rRNA gene sequence similarity and divergence[a] of each pair for *E. coli, Salmonella, Shigella, Enterobacter, Klebsiella*, and *Yersinia* calculated by DNASTAR

Strain no.	Strain name	% Similarity with strain no.													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*E. coli* ATCC 25922		99.6	97.4	97.2	96.5	95.4	99.6	99.9	99.8	95.0	95.9	95.4	95.1	93.0
2	*S. enterica* serovar Enteritidis ES22	2.6		97.6	97.4	97.4	97.1	96.7	96.9	96.9	95.1	96.9	95.1	95.5	91.6
3	*S. enterica* serovar Paratyphi A ATCC 54388	2.3	2.3		98.6	97.6	96.7	97.5	97.4	97.3	94.6	96.2	94.8	94.8	91.5
4	*S. enterica* serovar Paratyphi B P1	2.5	2.6	1.3		97.1	96.3	97.2	97.2	97.1	94.5	96.1	94.5	94.6	91.3
5	*S. enterica* serovar Typhi ATCC 19430	3.4	2.3	2.1	2.6		97.8	96.8	96.7	96.6	95.6	97.4	95.7	95.9	92.0
6	*S. enterica* serovar Typhimurium ATCC 13311	3.4	2.1	2.4	2.9	1.1		95.4	95.4	95.3	95.0	96.6	95.3	95.4	91.1
7	*S. boydii* ATCC 9207	0.4	2.8	2.1	2.4	3.1	3.4		99.7	99.7	94.6	95.5	95.1	94.7	92.8
8	*S. flexneri* ATCC 29903	0.1	2.6	2.2	2.4	3.3	3.4	0.3		99.9	94.9	95.7	95.3	95.0	93.0
9	*S. sonnei* ATCC 25931	0.2	2.6	2.3	2.5	3.4	3.4	0.3	0.1		94.8	95.6	95.1	94.8	93.0
10	*E. aerogenes* NCTC10006T	4.0	4.2	4.8	5.0	3.4	3.6	4.3	4.0	4.1		97.5	97.8	98.1	93.2
11	*E. cloacae* ATCC 13047T	3.3	2.6	3.1	3.4	1.6	1.9	3.6	3.3	3.4	2.2		97.2	98.3	92.1
12	*K. oxytoca* ATCC 13182T	3.8	4.4	4.8	5.1	3.4	3.4	3.9	3.8	3.9	1.8	2.6		97.4	92.3
13	*K. pneumoniae* ATCC 13883	4.0	3.9	4.5	4.8	3.1	3.1	4.2	4.0	4.0	1.5	1.5	2.6		93.0
14	*Y. enterocolitica* ATCC 9610	5.6	6.7	6.9	7.1	6.8	6.6	5.9	5.6	5.7	4.4	6.2	5.7	4.9	

[a] Percent divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign (DNASTAR). Percent similarity compares sequences directly without accounting for phylogenetic relationships.

Shigella, *Salmonella*, and *E. coli*. Using PCR, we amplified the *gyrB* regions (about 1.2 kb) of bacterial strains isolated from clinical specimens. This region was sequenced directly, and the results were used to compile phylogenetic relationships for the bacterial strains. In this study, we present a new phylogenetic analysis of *Shigella*, *Salmonella*, and *E. coli* determined from the *gyrB* gene region and compare the results with those of phylogenetic analysis by 16S rRNA.

MATERIALS AND METHODS

Bacterial strains and clinical specimens. The strains used in this study originated from reference collections (American Type Culture Collection and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) or were clinical isolates. Two hundred strains were cultured from stool specimens from patients with diarrhea collected from different regions in Japan during the period between April 1999 and May 2000. The serogroups of these 200 strains were identified by using an agglutination kit (Denka Seiken Co., Ltd., Tokyo, Japan), and the strains were numbered successively within each serogroup, for example, *S. sonnei* P1 (P for patient), P2, etc. Screening for *E. coli* O157:H7 was performed by culture on sorbitol MacConkey agar (Becton Dickinson and Company, Sparks, Md.).

Preparation of chromosomal DNA. The bacterial strains used in this study were obtained from clinical specimens and were cultured by standard methods. To isolate chromosomal DNA, one or two freshly grown colonies of bacteria were scraped into a 1.5-ml Eppendorf tube and resuspended in 500 μl of sterile water. The bacterial suspension was then boiled (at 100°C for 10 min) to release the DNA.

PCR. Chromosomal DNAs were amplified by PCR in a thermocycler 480 (Perkin-Elmer Co., Norwalk, Conn.). PCR was performed in a total volume of 100 μl with 5 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 200 mM (each) deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 10 pM primer UP1 (5′-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAA RTTYGA-3′), and 10 pM primer UP2r (5′-AGCAGGGTACGGATGTGCGA GCCRTCNACRTCNGCRTCNGTCAT-3′) (36). A 5-μl bacterial sample was added to the PCR solution, which underwent an initial denaturation step of 95°C for 5 min before 30 cycles of 96°C for 1 min, 63°C for 1 min, 72°C for 1 min, and then a final step of 72°C for 7 min for the last cycle. The PCR products were analyzed by 3% agarose gel electrophoresis.

Sequencing of *gyrB* genes. DNA sequencing was performed by the dideoxy chain termination method using a *Taq* DyeDeoxy Terminator cycle-sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. PCR fragments were determined by using the sequencing primers UP1S (5′-GAAGTCATCATGACCGTTCTGCA-3′) and UP2Sr (5′-AGCAGG GTACGGATGTGCGAGCC-3′) (36). Sequence reactions were analyzed on a PRISM 310 genetic analyzer (Applied Biosystems).

Phylogenetic analysis. The phylogenetic data described below were obtained by alignment and phylogenetic analysis of the bacterial sequences. The nucleotide sequences of 16S rRNA and *gyrB* were aligned by using the CLUSTAL V computer program (13). A neighbor-joining analysis (25) was used to reconstruct phylogenetic trees with the DNASTAR computer program (DNASTAR Inc., Madison, Wis.).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper appear in the GenBank and DDBJ nucleotide sequence databases with the following accession numbers: 16S rRNA genes, X80724, U90318, U88546, AF057362, Z47544, X80681, X96965, X96963, X96964, M59292, AJ251468, AJ251469, AF129440, and AF130981; *gyrB* genes, AB083821 to AB084027.

RESULTS

Phylogenetic analysis and genetic distance of 16S rRNA. Data for the phylogenetic analysis were obtained from sequences contained in the GenBank nucleotide sequence database (17). The following strains were examined: *E. coli* (ATCC 25922), *S. enterica* subsp. *enterica* serovar Enteritidis (SE22), *S. enterica* subsp. *enterica* serovar Paratyphi A (ATCC 54388), *S. enterica* subsp. *enterica* serovar Paratyphi B, *S. enterica* subsp. *enterica* serovar Typhi (ATCC 19430), *S. enterica* subsp. *enterica* serovar Typhimurium (ATCC 13311), *S. boydii* (ATCC 9027), *S. flexneri* (ATCC 29903), *S. sonnei* (ATCC 25931), *Yersinia enterocolitica* (ATCC 9610), *Enterobacter aerogenes* (NCTC10006T), *Enterobacter cloacae* (ATCC 13047T), *Klebsiella oxytoca* (ATCC 13182T), and *Klebsiella pneumoniae* (ATCC 13883). Alignment of the 16S rRNA nucleotide sequence, adjusted to 1,435 bases, was performed by the computer program MegAlign (DNASTAR Inc.). Table 1 shows the percent nucleotide divergence and similarity of 16S rRNA for *E. coli*, *Shigella*, *Salmonella, Enterobacter*, and *Klebsiella*, with *Yersinia* as an outgroup. *S. sonnei* and *S. flexneri* have 99.9% similarity to each other; 99.9 and 99.8%, respectively, to *E. coli*; and 99.7% to *S. boydii*. These results indicate that *S. flexneri* and *S. sonnei* are more closely related to *E. coli* than to *S. boydii*. Figure 1 shows the phylogenetic tree for these strains on the basis of their 16S rRNAs. In this tree, *Salmonella* subspe-

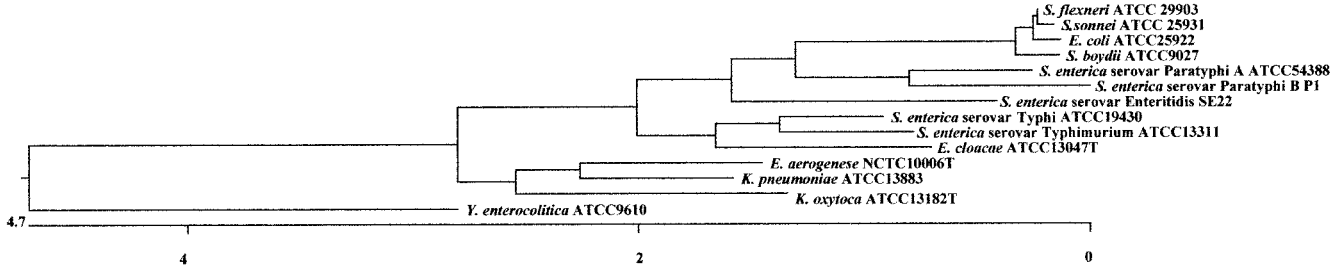


FIG. 1. Phylogenetic tree based on the nucleotide sequences of 16S rRNA genes. The 16S rRNA sequences were adjusted to 1,435 bases, and the tree was constructed by the neighbor-joining method, using the computer program MegAlign (DNASTAR Inc.). The scale indicates the percentage of base difference (percent divergence). The sequence data for phylogenetic analysis were taken from the GenBank nucleotide sequence database for the following strains: *E. coli* (ATCC 25922), *S. enterica* serovar Enteritidis (SE22), *S. enterica* serovar Paratyphi A (ATCC 54388), *S. enterica* serovar Paratyphi B, *S. enterica* serovar Typhi (ATCC 19430), *S. enterica* serovar Typhimurium (ATCC 13311), *S. boydii* (ATCC 9027), *S. flexneri* (ATCC 29903), *S. sonnei* (ATCC 25931), *Y. enterocolitica* (ATCC 9610), *E. aerogenes* (NCTC10006T), *E. cloacae* (ATCC 13047T), *K. oxytoca* (ATCC 13182T), and *K. pneumoniae* (ATCC 13883).

cies strains were grouped into two clusters. The first cluster contained *S. enterica* serovar Paratyphi A, *S. enterica* serovar Paratyphi B, and *S. enterica* serovar Enteritidis, while the second cluster contained *S. enterica* serovar Typhi and *S. enterica* serovar Typhimurium.

Phylogenetic analysis and genetic distance of *gyrB* genes. Bacterial samples were subjected to PCR amplification of the *gyrB* gene region with degenerate primers. The amplified *gyrB* gene, a region of 1,171 bp, was sequenced from five different strains of *Salmonella*, three different strains of *Shigella*, two different strains of *Enterobacter*, one strain of *E. coli*, two different strains of *Klebsiella*, and a strain *Yersinia enterocolitica* as a control. The following strains were examined: *E. coli* (ATCC 25922), *S. enterica* serovar Enteritidis (isolate), *S. enterica* serovar Paratyphi A (isolate), *S. enterica* serovar Paratyphi B (ATCC 8759), *S. enterica* serovar Typhi (isolate), *S. enterica* serovar Typhimurium (ATCC 14028), *S. boydii* (isolate), *S. flexneri* (ATCC 12022), *S. sonnei* (ATCC 11060), *Y. enterocolitica* (ATCC 23715), *E. aerogenes* (ATCC 13048), *E. cloacae* (ATCC13047), *K. oxytoca* (isolate), and *K. pneumoniae* (isolate). Alignment of the *gyrB* nucleotide sequences, adjusted to 1,171 bases, was performed by the computer program MegAlign. Table 2 shows the percent nucleotide divergence and similarity of the *gyrB* gene for *E. coli*, *Shigella*, *Salmonella*, *Enterobacter*, and *Klebsiella*, with *Yersinia* as an outgroup. *S. sonnei* and *S. flexneri* have 98.4% similarity to each other; 98.1 and 97.8%, respectively, to *E. coli*; and 99.1 and 98.7%, respectively, to *S. boydii*. The percent divergence of *E. coli* from *Salmonella* is greater in the *gyrB* gene than in 16S rRNA. Figure 2 shows the phylogenetic tree for these species based on the *gyrB* gene sequence. As indicated in Fig. 2, bacteria of the same genus are located in the same cluster. In other words, *S. enterica* serovar Enteritidis, *S. enterica* serovar Paratyphi A, *S. enterica* serovar Paratyphi B, *S. enterica* serovar Typhi, and *S. enterica* serovar Typhimurium form a cluster with *Salmonella* in contrast to the result with 16S rRNA (Fig. 1); similarly, *S. boydii*, *S. flexneri,* and *S. sonnei* form a cluster with *Shigella*.

Comparison of the genetic distance and the phylogenetic tree determined by 16S rRNA and *gyrB*. A direct comparison of the genetic distance and the phylogenetic tree determined by the 16S rRNA sequence with those determined by the *gyrB* sequence was not possible because the bacterial strains analyzed were not the same. However, the following observations could be made. First, the rate of genetic divergence of the *gyrB*

TABLE 2. *gyrB* gene sequence similarity and divergence[a] of each pair for *E. coli, Salmonella, Shigella, Enterobacter, Klebsiella*, and *Yersinia* calculated by DNASTAR

Strain no.	Strain name	% Similarity with strain no.													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*E. coli* ATCC25922		91.3	91.0	91.0	91.1	90.9	98.0	97.8	98.1	89.2	89.2	89.2	89.3	80.4
2	*S. enterica* serovar Enteritidis P1	9.2		98.2	98.1	97.9	98.0	91.4	91.1	91.2	89.7	89.1	89.5	90.4	79.4
3	*S. enterica* serovar Paratyphi A P 1	9.5	1.8		98.5	97.9	98.0	90.9	90.7	90.9	89.8	88.9	89.6	90.2	79.2
4	*S. enterica* serovar Paratyphi B ATCC 8759	9.5	1.9	1.5		98.9	99.2	90.9	91.1	90.9	90.3	89.1	89.7	90.6	79.3
5	*S. enterica* serovar Typhi P1	9.4	2.2	2.1	1.1		99.0	90.7	90.9	90.7	90.2	88.8	89.7	90.7	79.8
6	*S. enterica* serovar Typhimurium ATCC 14028	9.6	2.0	1.9	0.8	1.0		90.9	91.1	90.9	90.3	89.5	89.5	90.8	79.7
7	*S. boydii* P1	2.0	9.1	9.6	9.6	9.9	9.7		98.7	99.1	89.5	89.2	89.0	89.1	80.4
8	*S. flexneri* ATCC 12022	2.3	9.5	10.0	9.5	9.7	9.5	1.3		98.4	89.5	89.2	89.3	89.0	80.8
9	*S. sonnei* ATCC 11060	1.9	9.4	9.6	9.6	9.9	9.7	0.9	1.6		89.5	89.4	88.9	89.2	80.5
10	*E. aerogenes* ATCC 13048	11.7	11.1	10.8	10.4	10.4	10.3	11.4	11.3	11.4		88.3	91.3	93.4	79.2
11	*E. cloacae* ATCC 13047T	11.4	11.9	12.0	11.9	12.2	11.4	11.4	11.5	11.2	12.7		87.8	87.6	80.0
12	*K. oxytoca* P1	11.4	11.2	11.0	11.0	11.0	11.2	11.6	11.3	11.6	9.3	13.3		89.7	79.5
13	*K. pneumoniae* P1	11.6	10.4	10.5	10.1	10.0	9.9	11.9	11.8	11.7	6.8	13.5	11.2		79.0
14	*Y. enterocolitica* ATCC 23715	21.2	22.9	23.3	23.3	22.4	22.5	21.6	21.1	21.3	23.3	22.1	22.7	23.7	

[a] Percent divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign (DNASTAR). Percent similarity compares sequences directly without accounting for phylogenetic relationships.

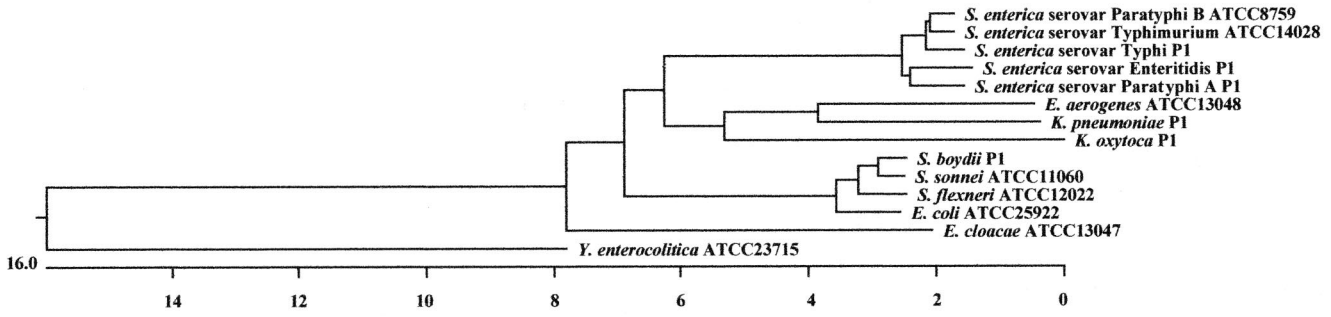


FIG. 2. Phylogenetic tree based on the nucleotide sequences of *gyrB* genes. The *gyrB* nucleotide sequences were adjusted to 1,171 bases, and the tree was constructed by the neighbor-joining method, using the computer program MegAlign (DNASTAR Inc.). The scale indicates the percentage of base difference (percent divergence). The mean sequence obtained from direct sequencing of PCR products was used for the following strains: *E. coli* (ATCC 25922), *S. enterica* serovar Enteritidis (isolate), *S. enterica* serovar Paratyphi A (isolate), *S. enterica* serovar Paratyphi B (ATCC 8759), *S. enterica* serovar Typhi (isolate), *S. enterica* serovar Typhimurium (ATCC 14028), *S. boydii* (isolate), *S. flexneri* (ATCC 12022), *S. sonnei* (ATCC 11060), *Y. enterocolitica* (ATCC 23715), *E. aerogenes* (ATCC 13048), *E. cloacae* (ATCC 13047), *K. oxytoca* (isolate), and *K. pneumoniae* (isolate).

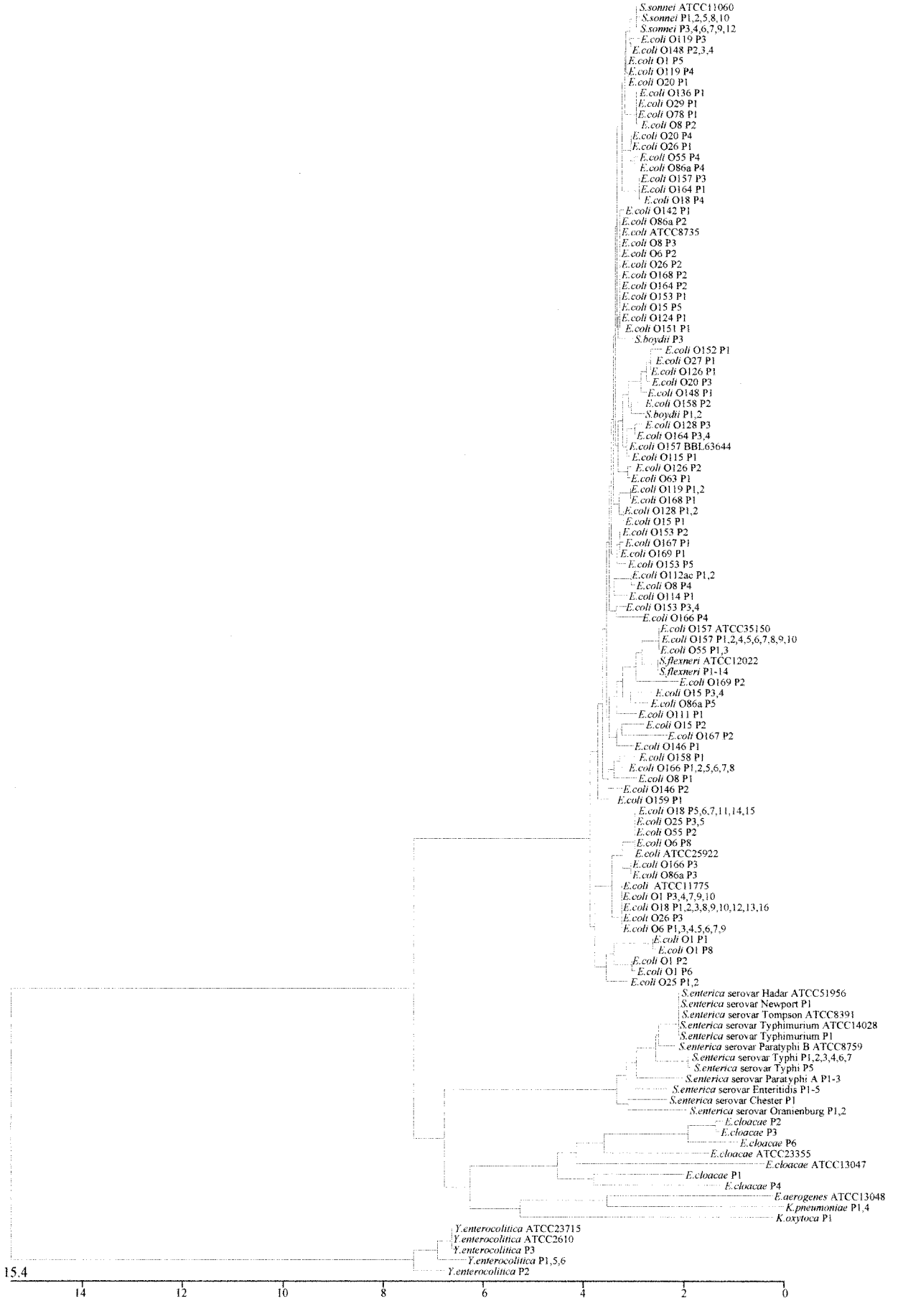
sequence differed greatly from that of the 16S rRNA sequence. For example, compared with 16S rRNA, the *gyrB* analysis shows there is a 4- to 10-fold increase in the length of branches between closely related species of *Shigella* and *E. coli*. Moreover, the topology of the phylogenetic tree based on the 16S rRNA sequence was quite different from that based on the *gyrB* sequence. Second, phylogenetic analysis using the *gyrB* nucleotide sequence determined a classification of the bacteria different from that determined by 16S rRNA analysis.

Phylogenetic analysis of *gyrB* genes from clinical specimens. For this study, data were gathered from the genes of 200 clinical specimens and the genes were sequenced. Alignment of the *gyr*B nucleotide sequences, adjusted to 1,171 bases, was done by the computer program MegAlign. Figure 3 shows the phylogenetic tree for these species based on the *gyrB* gene sequence. The numbers of bacterial strains sharing the same sequence in each group are indicated. For example, *S. enterica* serovar Enteritidis P1 to P5, five strains of *S. enterica* serovar Enteritidis, have identical *gyrB* sequences, which were isolated from five patients. On one hand, it has been reported that it is impossible to differentiate between *E. coli* and *Shigella* on the basis of 16S rRNA sequence analysis (7). However, even if some *Shigella* strains are distributed among *E. coli* strains, our results clearly show that *Shigella* spp. are not identical to *E. coli*. On the other hand, it was not possible to classify serogroups of *Salmonella* and *E. coli* by phylogenetic analysis of either the 16S rRNA or the *gyrB* gene. *E. coli* isolates with the same O serogroups appear in several different clusters. For example, *E. coli* O157 isolates are found in three different clusters. It is interesting that *E. coli* O157 BBL63644 was identified as a sorbitol-fermenting EHEC isolate, representing the H^- serogroup (5, 23). In general, serogroup does not correspond to genotype. Indeed, our analysis showed that serogroup does not correspond to genotype.

DISCUSSION

Phylogenetic-tree analysis is often used as a method to classify organisms. Various genes have been examined for the analysis of phylogenetic relationships of *Salmonella*, *Shigella*, *and E. coli* (1, 2, 16, 21). In general, 16S rRNA is most frequently used for such analyses; however, phylogenetic relationships between closely related species of *Shigella* and *E. coli* are weakly defined by this approach, as the 16S rRNA sequences of bacteria contain highly conserved regions. In terms of cell biology, *Shigella* spp. are similar to *E. coli. Shigella* strains are in reality clones of *E. coli* (14, 28) and are believed to have emerged relatively recently (21, 32). In our phylogenetic-tree analysis of 16S rRNA, *S. sonnei* and *S. flexneri* were found to have 99.9% similarity to each other and 99.9 and 99.8% similarity, respectively, to *E. coli* but only 99.7% similarity to *S. boydii* (Table 1). The percent divergences of *E. coli* from *S. sonnei*, *S. flexneri*, and *S. boydii* were 0.2, 0.1, and 0.4%, respectively. These data are in accordance with the previous study using the 16S rRNA gene sequence (34), indicating close relationships among these bacteria. In Fig. 3., *S. sonnei* (15 specimens) and *S. flexneri* (17 specimens) are grouped together, but the groups are scattered among *E. coli* strains. This result gives results comparable to those of studies with other genes (14, 20, 28), supporting the idea that *Shigella* strains are actually clones of *E. coli*. The divergence values within or between species are smaller in 16S rRNA analysis than in previous studies using several housekeeping and invasion genes (1, 2, 16, 19, 20, 26). For example, in the nucleotide sequence of the isocitrate dehydrogenase gene, pairs of *E. coli* strains differed by 5.6% on average and pairs of *E. coli* and *S. enterica* strains differed by 13.3% (30). Although the 16S rRNA gene is preferred for phylogenetic studies because of its attributes, such as little evidence for its lateral transfer (27), the

FIG. 3. Phylogenetic tree based on the nucleotide sequences of *gyrB* genes taken from 200 clinical specimens. The *gyrB* nucleotide sequences were adjusted to 1,171 bases, and the tree was constructed by the neighbor-joining method, using the computer program MegAlign (DNASTAR Inc.). The scale indicates the percentage of base difference (percent divergence). Amplified *gyrB* genes from five subspecies of *Salmonella*, three species of *Shigella*, two species of *Enterobacter*, one species of *E. coli*, two species of *Klebsiella*, and *Yersinia* species used as an outgroup were sequenced. Bacterial strains of the same species with the same gene sequence are displayed in one group; for example, the cluster labeled *S. enterica* serovar Enteritidis P1-5 includes five strains of *S. enterica* serovar Enteritidis with identical *gyrB* sequences which were isolated from patients.


S.sonnei ATCC11060
S.sonnei P1,2,5,8,10
S.sonnei P3,4,6,7,9,12
E.coli O119 P3
E.coli O148 P2,3,4
E.coli O1 P5
E.coli O119 P4
E.coli O20 P1
E.coli O136 P1
E.coli O29 P1
E.coli O78 P1
E.coli O8 P2
E.coli O20 P4
E.coli O26 P1
E.coli O55 P4
E.coli O86a P4
E.coli O157 P3
E.coli O164 P1
E.coli O18 P4
E.coli O142 P1
E.coli O86a P2
E.coli ATCC8735
E.coli O8 P3
E.coli O6 P2
E.coli O26 P2
E.coli O168 P2
E.coli O164 P2
E.coli O153 P1
E.coli O15 P5
E.coli O124 P1
E.coli O151 P1
S.boydii P3
E.coli O152 P1
E.coli O27 P1
E.coli O126 P1
E.coli O20 P3
E.coli O148 P1
E.coli O158 P2
S.boydii P1,2
E.coli O128 P3
E.coli O164 P3,4
E.coli O157 BBL63644
E.coli O115 P1
E.coli O126 P2
E.coli O63 P1
E.coli O119 P1,2
E.coli O168 P1
E.coli O128 P1,2
E.coli O15 P1
E.coli O153 P2
E.coli O167 P1
E.coli O169 P1
E.coli O153 P5
E.coli O112ac P1,2
E.coli O8 P4
E.coli O114 P1
E.coli O153 P3,4
E.coli O166 P4
E.coli O157 ATCC35150
E.coli O157 P1,2,4,5,6,7,8,9,10
E.coli O55 P1,3
S.flexneri ATCC12022
S.flexneri P1-14
E.coli O169 P2
E.coli O15 P3,4
E.coli O86a P5
E.coli O111 P1
E.coli O15 P2
E.coli O167 P2
E.coli O146 P1
E.coli O158 P1
E.coli O166 P1,2,5,6,7,8
E.coli O8 P1
E.coli O146 P2
E.coli O159 P1
E.coli O18 P5,6,7,11,14,15
E.coli O25 P3,5
E.coli O55 P2
E.coli O6 P8
E.coli ATCC25922
E.coli O166 P3
E.coli O86a P3
E.coli ATCC11775
E.coli O1 P3,4,7,9,10
E.coli O18 P1,2,3,8,9,10,12,13,16
E.coli O26 P3
E.coli O6 P1,3,4,5,6,7,9
E.coli O1 P1
E.coli O1 P8
E.coli O1 P2
E.coli O1 P6
E.coli O25 P1,2
S.enterica serovar Hadar ATCC51956
S.enterica serovar Newport P1
S.enterica serovar Tompson ATCC8391
S.enterica serovar Typhimurium ATCC14028
S.enterica serovar Typhimurium P1
S.enterica serovar Paratyphi B ATCC8759
S.enterica serovar Typhi P1,2,3,4,6,7
S.enterica serovar Typhi P5
S.enterica serovar Paratyphi A P1-3
S.enterica serovar Enteritidis P1-5
S.enterica serovar Chester P1
S.enterica serovar Oranienburg P1,2
E.cloacae P2
E.cloacae P3
E.cloacae P6
E.cloacae ATCC23355
E.cloacae ATCC13047
E.cloacae P1
E.cloacae P4
E.aerogenes ATCC13048
K.pneumoniae P1,4
K.oxytoca P1
Y.enterocolitica ATCC23715
Y.enterocolitica ATCC2610
Y.enterocolitica P3
Y.enterocolitica P1,5,6
Y.enterocolitica P2
15.4
14
12
10
8
6
4
2
0

sequence variation of 16S rRNA is not as high as those of other genes in numerous studies.

In the phylogenetic-tree analysis of *gyrB*, *S. sonnei* and *S. flexneri* were found to have 98.4% similarity to each other, 98.1 and 97.8% similarity to *E. coli*, and 99.1 and 98.7% similarity to *S. boydii*, respectively (Table 2). The percent divergences of *E. coli* from *S. sonnei*, *S. flexneri*, and *S. boydii* were 1.9, 2.3, and 2.0%, respectively. The divergence values are significantly improved in *gyrB* analysis compared with 16S rRNA analysis. It is reported that when 20 *Pseudomonas* strains were analyzed by using the nucleotide sequences of *gyrB* and the genes for 16S rRNA and RNA polymerase σ^{70} factor (*rpoD*), the percent divergences of *gyrB* were larger than those of the other genes (38). Like the 16S rRNA gene, the *gyrB* gene does not appear to be frequently horizontally transmitted and can be found in most, if not all, bacterial species (36, 38). Many genes, especially those for catabolism, are known to spread horizontally among different bacterial species, and they cannot be used to trace the evolutionary records of host bacteria (37).

A comparison of Fig. 2 with Fig. 1 highlights the different patterns of bacterial divergence determined through analysis of *gyrB* genes and 16S rRNA. In the phylogenetic analysis using 16S rRNA, bacteria belonging to the same genus were not always located in the same cluster; by contrast, in the phylogenetic analysis using the *gyrB* gene, bacteria of the same genus were clustered together. In other words, *S. enterica* serovar Enteritidis, *S. enterica* serovar Paratyphi A, *S. enterica* serovar Paratyphi B, *S. enterica* serovar Typhi, and *S. enterica* serovar Typhimurium were located in a cluster with *Salmonella*. Similarly, *S. boydii*, *S. flexneri*, and *S. sonnei* were located in a cluster with *Shigella*. In addition, the rate of base substitution was greater for the *gyrB* sequence than for the 16S rRNA sequence (9, 36, 38). Thus, certain bacterial strains were classified differently under the two phylogenetic analyses.

It seems likely that phylogenetic analysis using the *gyrB* gene sequence will be able to classify some bacteria that cannot be classified by their 16S rRNA sequences. Cilia et al. (8) have reported that 16S rRNA sequences cannot be used to derive phylogenetic-tree analyses among closely related bacteria, for example, *Shigella* and *E. coli*, owing to the similarity in these gene regions. However, our results indicate that such closely related bacteria might be classified by *gyrB* analysis. 23S rRNA sequence analysis (7) could provide phylogenetic information at the subspecies level for *Salmonella*, but *gyrB* analysis generally shows sharper separations.

Approximately 2,000 serotypes of *S. enterica* can cause sickness in humans, such as *S. enterica* serovar Typhi, which causes enteric (typhoid) fever and is pathogenic only in humans, and *S. enterica* serovar Typhimurium, which causes gastroenteritis and is pathogenic for several mammalian species. Currently, salmonellosis is classified clinically according to the symptoms (typhoid fever and paratyphoid) and/or according to the serotype, especially for non-typhoid fever types of salmonellosis. However, as serotype does not necessarily agree with genotype, it would be useful to have another means, such as *gyrB* gene analysis, to classify the subspecies of *S. enterica*. In contrast to serotype assays, genotype assays for pathogenic bacteria may be expensive and require technical expertise, but they could detect a wider range of bacterial strains and increase specificity (18), which is useful when cross-reaction is observed with serotyping reagents.

As shown in Fig. 3, *E. coli* isolates with the same O serogroups appear in several different clusters. Our results also show a mosaic relationship among pathotypes classified by O serogroup and genotypes classified by *gyrB* region. For example, *E. coli* isolates with O serogroups associated with EPEC and EHEC appear in different clusters or different pathotypes are grouped in one cluster. These results indicate lateral transfer of genes for O antigens (20, 31), as well as genes for H antigens (23), among *E. coli* strains.

The rate of evolution of the *gyrB* genetic region is higher than that of the 16S rRNA region, and the *gyrB* genetic region is found in all bacterial species. We believe that the *gyrB* region will have high reliability for identifying pathogenic bacteria. Although the 16S rRNA sequence method is a highly accurate and rapid method for identifying most bacteria to the genus level (33), the *gyrB* sequence method might be more useful for identifying bacteria to the species level. In practical terms, this means that whereas it is easy to amplify the 16S rRNA sequence because it is highly conserved across bacteria, it is harder to amplify the *gyrB* region because of its variability. However, the primer set developed by Yamamoto and Harayama (36) for amplification of the bacterial *gyrB* region by PCR simplifies the rapid amplification of this region. It is known that the amplified *gyrB* region we used includes the region involving quinolone resistance (11, 39). However, the 200 clinical specimens in our study do not include quinolone-resistant strains (data not shown), indicating that this resistance does not influence the interpretation of our results.

In summary, we have shown that *gyrB* sequence analysis is a fruitful approach to determine the phylogenetic relationships of bacteria and may be an alternative to 16S rRNA analysis. In particular, *gyrB* analysis of bacteria is an effective means to classify closely related species. Further research on *gyrB* sequence analysis will clarify in more detail the classification of bacterial species.

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