JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2000, p. 3991–3993 Vol. 38, No. 11
0095-1137/00/$04.00+0

Diversity of Domain V of 23S rRNA Gene Sequence in Different *Enterococcus* Species

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Received 31 May 2000/Returned for modification 11 July 2000/Accepted 8 September 2000

The highly conserved central loop of domain V of 23S RNA (nucleotides 2042 to 2628; *Escherichia coli* numbering) is implicated in peptidyltransferase activity and represents one of the target sites for macrolide, lincosamide, and streptogramin B antibiotics. DNA encoding domain V (590 bp) of several species of *Enterococcus* was amplified by PCR. Twenty enterococcal isolates were tested, including *Enterococcus faecium* (six isolates), *Enterococcus faecalis*, *Enterococcus avium*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus casseliflavus* (two isolates of each), and *Enterococcus raffinosus*, *Enterococcus mundtii*, *Enterococcus malodoratus*, and *Enterococcus hirae* (one isolate of each). For all isolates, species identification by biochemical testing was corroborated by 16S rRNA gene sequencing. The sequence of domain V of the 23S rRNA gene from *E. faecium* and *E. faecalis* differed from those of all other enterococci. The domain V sequences of *E. durans* and *E. hirae* were identical. This was also true for *E. gallinarum* and *E. casseliflavus*. *E. avium* differed from *E. casseliflavus* by 23 bases, from *E. durans* by 16 bases, and from *E. malodoratus* by 2 bases. *E. avium* differed from *E. raffinosus* by one base. Despite the fact that domain V is considered to be highly conserved, substantial differences were identified between several enterococcal species.

The first complete nucleotide sequence of the 23S rRNA gene from an enterococcus was recently published (8). An earlier report described the application of a 23S rRNA gene-targeted oligonucleotide probe specific for enterococci to detect these organisms in water samples with results superior to those obtained with a common biochemical test panel (3). The highly conserved central loop of domain V of 23S RNA (nucleotides 2042 to 2628; *Escherichia coli* numbering) is involved in the peptidyltransferase center (16) and represents a target site for multiple antibiotics, including chloramphenicol, macrolide-lincosamide-streptogramin B antibiotics (7), and oxazolidinones (6). Mutations in this area have been associated with macrolide resistance in clinical isolates of *Helicobacter pylori*, (15), *Mycobacterium avium* (9), *Propionibacterium acnes* (12), and very recently in *Streptococcus pneumoniae* (14).

To date, molecular analysis of nucleotide sequences in this area has not been performed systematically for various enterococcal species. Domain V sequence data are important for the study of antibiotic interactions at this particular site of the 23S rRNA, as they might relate to resistance in clinical isolates of enterococci. Furthermore, sequence data could potentially enhance understanding of the phylogenetic relationship between clinically relevant enterococcal species. The objective of this study was to determine domain V 23S rRNA gene sequences from several isolates of the enterococcal species *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus avium*, *Enterococcus durans*, *Enterococcus raffinosus*, *Enterococcus gallinarum*, *Enterococcus mundtii*, *Enterococcus malodoratus*, and *Enterococcus hirae* and to compare phylogenetic relationships based on these sequences with those determined by analysis of 16S rRNA genes.

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MATERIALS AND METHODS

Bacterial strains and identification. Six isolates of *E. faecium* (including ATCC 19434 and ATCC 51558); two isolates each of *E. faecalis* (including ATCC 29212), *E. avium* (including ATCC 14025), *E. durans* (including ATCC 19432), and *E. casseliflavus* (including ATCC 25788); two clinical isolates of *E. gallinarum*; and one isolate each of *E. raffinosus*, *E. mundtii* (ATCC 43186), *E. malodoratus* (ATCC 43197), and *E. hirae* (ATCC 8043) were studied. Initial identification was accomplished by assessing biochemical properties (API 20 Strep system; bioMérieux Vitek, Inc., Hazelwood, Mo.), pigment production, and motility. Motility was assessed in motility agar tubes (Motility B medium; Remel, Lenexa, Kans.) that were incubated at 30°C for up to 72 h. The presence of the chromosomal aminoglycoside resistance determinant *aac(6′)-Ii* (specific to *E. faecium*) was detected by DNA probe analysis of lysed whole cells (1, 2) and was used to confirm the identification of *E. faecium*. Species assignment was confirmed by 16S rRNA gene sequence analysis based on published data (10).

PCR. Nucleotide sequences of domain V from *E. coli* (GenBank accession number AF053966) and *E. faecium* (GenBank accession number AJ007584) and rRNA homologues from the unedited genomic sequence of *E. faecalis* (provided by The Institute for Genomic Research website, http://www.tigr.org) were aligned. The alignment of amino acid sequences of *E. coli* and *E. faecalis* was performed using the Clustal method (4) with the MEGALIGN software program (DNASTAR, Inc., Madison, Wis.). PCR primers were designed based on conserved sequences to amplify domain V of both *E. faecium* and *E. faecalis*.

The selected primers consisted of the following nucleotides: A, 5′-TGG GCA CTG TCT CAA CGA-3′ (corresponding to *E. coli* bases 1984 to 2001) and B, 5′-GGA TAG GGA CCG AAC TGT CTC-3′ (corresponding to *E. coli* bases 2597 to 2617). After aligning all 16S rRNA gene sequences of enterococci available in GenBank, it was determined that sequencing of an area approximately 500 bp in length in the 5′ end of the 16S rRNA gene would adequately differentiate between enterococcal species (data not shown). For the 16S rRNA gene sequence, conserved areas were used to design the 5′ primer (GGC GTG CCT AAT ACA) and the 3′ primer (GTC TTT CGG TGC) corresponding to bases 13 to 27 and 484 to 495 of the 16S rRNA gene sequence of *E. faecium* (GenBank accession number AF070223).

PCR protocol. Bacterial colonies or genomic DNA served as the PCR template. PCR reagents were from the Perkin-Elmer GeneAmp PCR reagent kit (Foster City, Calif.). The PCR program consisted of a lysis and denaturation step of 5 min at 95°C, 30 cycles with a 30-s denaturation step at 94°C, a 30-s annealing step at 58°C, a 30-s extension at 72°C, and a final 10-min extension step at 72°C. Samples were held at 4°C until analysis. PCR products were separated in a 1% agarose gel with 150 V for 45 min, stained using ethidium bromide, and photographed under UV light.

Sequencing. Amplification products were either sequenced directly or first cloned into pCR 2.1 and transformed into One Shot-competent *E. coli* (TA Cloning kit; Invitrogen, Carlsbad, Calif.). Screening for transformants was done using Luria-Bertani agar plates containing ampicillin (50 μg/ml) and X-Gal

TABLE 1. Homology between various enterococci based on 23S rRNA gene sequencing data[a]

Organism	% Homology									
	E. avium	*E. casseliflavus*	*E. durans*	*E. faecalis*	*E. faecium*	*E. gallinarum*	*E. hirae*	*E. malodoratus*	*E. mundtii*	*E. raffinosus*
E. avium		91.4	94.4	94	94.7	91.4	94.4	99.3	95.7	99.7
E. casseliflavus			94.4	91.7	94.4	100	94.4	90.7	94.4	91.4
E. durans				94.4	99.7	94.4	100	94.4	98.3	94.7
E. faecalis					94.7	91.7	94.4	93.4	94	93.7
E. faecium						94.4	99.7	94.7	98.3	94.4
E. gallinarum							94.4	90.7	94.4	91.4
E. hirae								94.4	98.3	94.7
E. malodoratus									95.7	99
E. mundtii										95.7
E. raffinosus										

[a] Values represent percent homology between different sequences.

(5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (80 μg/ml). Sequencing was performed at the Molecular Biology Automated DNA sequencing facility, Harvard Institutes of Medicine (Boston, Mass.). The Clustal method (4) was used to perform alignments of DNA sequences with the MEGALIGN software program as well as the SEQMAN software program (DNASTAR, Inc.).

Probe preparation. The amplification products obtained by PCR of *E. faecium* and *E. faecalis* were cloned and labeled with digoxigenin-dUTP (DIG/Genius system; Boehringer Mannheim, Indianapolis, Ind.) for use as gene probes for rRNA operons.

Southern transfers and DNA hybridization. Genomic DNA of one strain of *E. faecium* (ATCC 51558) and one strain of *E. faecalis* (ATCC 29212) was digested with *Xba*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Pvu*II, or *Xba*I and *Pvu*II (Promega, Inc., Madison, Wis.); electrophoresed; and transferred to nylon membranes in preparation for DNA probing.

Nucleotide sequence accession numbers. All the nucleotide sequences have been submitted to GenBank (accession numbers AF273482 to AF273491).

RESULTS

Species identification of the isolates tested was initially performed by biochemical testing and was uniformly corroborated by previously published 16S rRNA gene sequences (10). All isolates of any one species yielded identical domain V rRNA gene sequences. The 23S rRNA gene sequence of *E. faecium* differed from those of all other enterococci by at least one nucleotide base (Table 1). The sequence of *E. faecalis* was also unique and differed by 14 bases from that of *E. faecium* strains. *E. durans* and *E. hirae* shared the same domain V nucleotide sequence. This was true for *E. casseliflavus* and *E. gallinarum* as well. *E. raffinosus* differed by one base from *E. avium*. Homology values (percent similarity between the various sequences) are presented in Table 1.

An alignment between the 23S rRNA gene sequence data of the various enterococci showed that the specific area where differences were found among different enterococci corresponds to *E. coli* bases 2080 to 2380 (data available on request). The remainder of the sequences (corresponding to *E. coli* bases 1984 to 2080 and 2380 to 2617) were identical to previously published sequencing data for 23S RNA for *E. faecium* (8).

A phylogenetic distance matrix tree constructed by the DNASTAR MEGALIGN software program is shown in Fig. 1 and was similar to one based on 16S rRNA gene sequences (10). Examining genomic DNA (digested with *Xba*I and *Pvu*II) with a probe generated against the domain V area sequence revealed the presence of at least five homologous bands in both the *E. faecium* and *E. faecalis* genomes (data not shown), in concordance with findings published by other investigators (13).

DISCUSSION

It has been suggested that sequencing data for the 23S rRNA molecule may inherently have more discriminatory power than 16S data to provide phylogenetic information (5), but data on enterococcal 23S rRNA gene sequences supporting this conjecture are currently lacking. Our study provides information that may be useful in the design of strategies incorporating nucleotide sequence analyses to differentiate among enterococcal species. We identified several differences between various enterococci in the highly conserved area of domain V of the large ribosomal subunit. A phylogenetic tree constructed from these data was similar to the one based on 16S rRNA gene data. Nevertheless, this approach did not distinguish between the closely related species *E. hirae* and *E. durans* or *E. gallinarum* and *E. casseliflavus* and thus has less discriminatory power for these sequences than 16S RNA sequencing. Phylogenetic trees are dynamic constructs that may change with the addition of new sequence data (5). Recently, the *sodA* gene encoding the manganese-dependent superoxide dismutase was suggested to be a more discriminative target sequence than the 16S rRNA gene in differentiating closely related enterococcal species (11). Since it is unlikely that functionally independent genes (i.e., *sodA* and 16S RNA) have preserved information from the same periods during evolution (5), it seems likely that sequencing of multiple different genes would provide more efficient discrimination between different species. We speculate that 23S rRNA gene sequence data from the entire molecule may provide that level of discrimination as well.

Furthermore, the DNA sequence data that we have provided spans critical areas of antibiotic-ribosomal interactions relating to antibiotic resistance. It is likely that mutations in

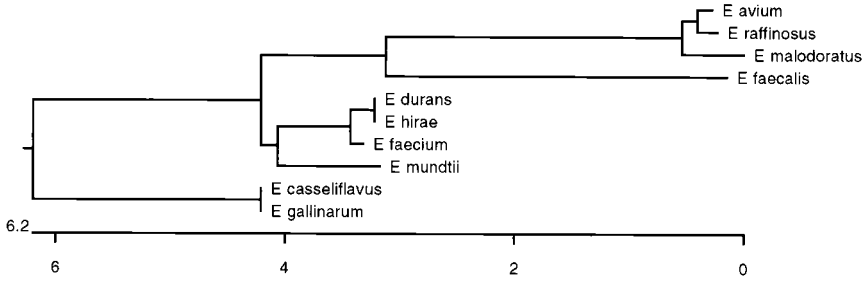

FIG. 1. Phylogenetic relationship derived from sequence homology data of 23S rRNA genes. Phylogenies are rooted, assuming a biological clock. The length of each pair of branches represents the distance between sequence pairs. The units on the horizontal axis indicate the number of substitution events.

this area can contribute to antibiotic resistance in enterococci as well (14). Mutations in codons 2058 and 2059 (*E. coli* numbering) have been associated with macrolide resistance in other bacterial species (12, 15). Technical difficulties associated with the study of bacteria with more than two operons will be overcome when full genomic data become available for each species.

A coordinated scientific effort, taking into account the presence of multiple ribosomal operons, and further interactions of the domain V area with other large ribosomal-subunit domains and proteins will allow further exploration of these mechanisms and contribute to our understanding of antimicrobial resistance.

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