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Length Polymorphisms in tRNA Intergenic Spacers Detected by Using the Polymerase Chain Reaction Can Distinguish Streptococcal Strains and Species

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Intergenic tRNA spacers from strains of streptococcal groups A, B, and G were amplified by using the polymerase chain reaction (PCR) at low stringency with consensus tRNA gene primers. Cloning and sequencing showed that many of the homologous intergenic spacers differed in length between species. The sequences of the tRNA genes that flank these polymorphic spacers were determined and used to synthesize fully complementary primers. With these primers at high stringency, PCR products which varied in lengths from 53 to 71 bp, depending on the species or strain, were obtained from streptococcal DNAs, even in the presence of a 1,000-fold mass excess of human DNA. PCR products, the lengths of which could also be used for classification, were obtained at high stringency from a few genera closely related to *Streptococcus*. No products were obtained from genomic DNAs from more distantly related genera. Production of species- or strain-specific tRNA intergenic length polymorphisms with primers that generate characteristic products from a variety of species within the same genus should be applicable to many organisms, including those that would otherwise be difficult to culture or identify.

Polymerase chain reaction (PCR)-based methods for the detection of bacteria can generally be used to detect DNA from a single species or from a set of closely related strains by producing a defined DNA product. This has been demonstrated with, for example, *Mycoplasma pneumoniae* (1), *Rickettsia rickettsii* (13, 21), *Borrelia burgdorferi* (12), *Listeria monocytogenes* (2), and *Treponema pallidum* (3). In general, primers directed toward arbitrarily chosen sequences, such as a random clone or an open reading frame, result in a PCR product only if the relevant species is present in the sample, and they result in no product when the species is absent. However, certain highly conserved sequences, e.g., rRNA genes and tRNA genes, can be used to design primers that allow PCR amplification of intergenic regions from many phylogenetically related groups. The length of the resultant PCR product, rather than its presence or absence, is characteristic of the group. Such primers can be derived from tRNA genes for a number of reasons. First, tRNA genes are highly conserved among species within a genus, so primers that will allow a PCR product to be amplified from practically all members of a genus can usually be devised. Second, bacterial tRNA genes are generally clustered in cistrons. For instance, *Bacillus subtilis* has a cistron of 21 tRNA genes (14). While tRNA genes themselves are highly conserved, the lengths of tRNA intergenic spacers vary considerably, ranging, for example, from 2 to 35 bp in *B. subtilis* (14) and from 2 to 208 bp in *Escherichia coli* (8). Third, the order of tRNA genes within a cistron appears to be highly conserved within a genus. The tandem arrangements of tRNA gene clusters allow them to be explored very efficiently for interspecific intergenic length polymorphisms (ILPs) by using low-stringency PCR amplification with consensus primers derived from tRNA gene sequences (tDNA [i.e., the gene encoding tRNA]-PCR; 16). tDNA-PCR showed that the lengths of tRNA intergenic regions generally evolve at rates that allow different species within a single genus to be distinguished from each other (16). Once a sufficiently polymorphic intergenic region has been identified by tDNA-PCR, primers that are directed toward the highly conserved flanking tRNA genes can be used to amplify the polymorphic region in any organism that is sufficiently closely related. In bacterial systematics, this generally corresponds to the genus level. We wished to demonstrate this method for the development of diagnostically useful PCR primers for the genus *Streptococcus*.

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MATERIALS AND METHODS

Genomic DNAs. Strains supplied by S. Hollingshead (University of Alabama) included *Streptococcus pyogenes* group A, strains 52RS15 and Ti/195/2; *Streptococcus* sp. group B, strains B16501 and 501-1b; *Streptococcus* sp. group G, strains 1/E9 and 1/B1/A2; *Streptococcus mutans* T8; and *Enterococcus faecalis* OG1X. Staphylococcal strains, supplied by W. Kloos (North Carolina State University), included *Staphylococcus hominis* 27844 and 27846, *Staphylococcus warneri* CPB10E2 and GAD473, *Staphylococcus aureus* 6538 and Sau3A, *Staphylococcus haemolyticus* AW263 and 29970, and *Staphylococcus cohnii* CM89 and SS521. All other strains were obtained through the American Type Culture Collection.

Genomic DNAs were prepared from late-log-phase cultures. Cell walls were treated with mutanolysin (streptococci) or lysostaphin (staphylococci), and then they were incubated at 65°C in a solution containing 1 mg of proteinase K per ml, 100 mM EDTA, and 1% *N*-lauryl sarcosine for 2 h. DNA was purified by phenol extraction followed by chloroform extraction and isopropanol precipitation.

Bacillus globigii DNA was obtained from G. G. Wilson at New England Biolabs, Beverly, Mass. Human DNA was obtained from M. Perucho at the California Institute of Biological Research.

Oligonucleotide primers. The following primers were pur-

chased from Genosys (Houston, Tex.) or Stratagene (La Jolla, Calif.):

T5A	5'AGTCCGGTGCTCTAACCAACTGAG
T3B	5'AGGTCGCGGGTTCGAATCC
Uni	5'TTGTAAAACGACGGCCAG
Rev	5'GGAAACAGCTATGACCATGA
SpytiMet3	5'AAGGTCGTAGGTTCAAATCC
SpytPhe5	5'ACCAACTGAGCTACCGAGCC
T7	5'AATACGACTCACTATAG
T3	5'ATTAACCCTCACTAAAG

PCR. tDNA-PCR with consensus primers was performed according to the published protocol (16). Reaction mixtures (50 μl each) were prepared by using 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus or Stratagene), 1× *Taq* polymerase buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3], 1.5 mM $MgCl_2$), 0.2 mM each deoxynucleoside triphosphate (dNTP), 5 μCi of [α-^{32}P]dCTP, 0.5 μM each primer, and 10 ng of template DNA. The reaction mixture was cycled 40 times in a Perkin-Elmer Cetus 9600 thermocycler with the following temperature profile: 94°C for 30 s to denature, 50°C for 30 s for annealing of primer, and 72°C for 2 min for extension. The resulting products were resolved by electrophoresis with 5% acrylamide–50% urea in 1× Tris-borate-EDTA and visualized by autoradiography with Kodak X-Omat AR film with an intensifying screen at −70°C for 6 h. The products of tDNA-PCR can also be visualized on NuSeive agarose (FMC, Rockland, Maine) or native acrylamide gels by ethidium bromide staining.

The location of PCR products in the gel was determined by using radioactive ink spots to align the autoradiogram with the gel. The PCR products were cut out of the gel and placed in 50 μl of Tris-EDTA, and the DNA was eluted for 1 h at 65°C. A 1-μl sample of the eluate was reamplified by PCR with the same primers (see below).

High-stringency PCR amplification was done in 1× *Taq* polymerase buffer, 0.2 mM each dNTP, 0.5 μM each primer, 30 ng of template, and 1.25 U of *Taq* polymerase (in a total volume of 50 μl). Cycling parameters were 94°C (1 min), 60°C (1 min), and 72°C (2 min) for 30 cycles. For tDNA-ILPs, the cycling times can be shortened as follows if the expected product is short: 94°C (30 sec), 60°C (30 sec), and 72°C (60 sec) for 40 cycles.

Cloning. Gel-purified and reamplified PCR products were purified with GeneClean (Bio101, San Diego, Calif.). The DNA was cloned with 100 ng of pBSKII$^+$ digested with *Sma*I and T4 DNA ligase with 0.5 mM ATP and the recommended buffer. Clones were detected as white colonies on 2× yeast extract tryptone plates sprayed with the lactose analog isopropyl-β-D-thiogalactopyranoside and the colorimetric indicator of β-galactosidase activity X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Ten colonies were picked and boiled in 100 μl of water, and 10 μl was amplified in a 50-μl PCR reaction mixture with the Universal (Uni) and reverse (Rev) sequencing primers. The products were electrophoresed through 1.5% agarose (1× Tris-borate-EDTA) to determine which recombinant plasmids contained inserts of the correct size.

Sequencing. A 5-μl volume of PCR product from the Uni-Rev amplification was asymmetrically reamplified in each direction by using each of the two sequencing primers separately. The largely single-stranded DNA that resulted from asymmetric amplification was then sequenced with a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio) with the pBSKII$^+$ T7 primer for asymmetrically Uni-amplified material and the T3 primer for asymmetrically Rev-amplified material. The sequences were resolved on 5% polyacrylamide–50% urea gels followed by fixing, drying, and autoradiography.

Nucleotide sequence accession number. Gene sequences have been placed in GenBank/EMBL under accession numbers M92070 (group A streptococci), M92071 (group B streptococci), and M92072 (group G streptococci).

RESULTS

Amplification with consensus primers. Two primers for low-stringency PCR were derived from a consensus of *Bacillus* tRNA genes in a manner presented earlier (16). These primers were located within the tRNA consensus sequence and faced outward into the intergenic spacer region. The consensus primers were recessed by about 15 bases from the 5' and 3' ends of the tRNA gene consensus sequence, so that this portion of the tRNA would be amplified along with the intergenic spacer during subsequent PCR steps. PCR amplification of several divergent streptococcal DNA templates with these primers at low stringency resulted in fingerprints that displayed the distances between those tRNA genes that, by chance, amplified the most efficiently (Fig. 1). Fingerprinting with consensus primers in this way, called tDNA-PCR (16), rarely uncovers differences between strains within a species. For instance, in experiments not shown, 13 strains of group A with six different M types were compared and there were no intragroup differences. However, when 27 streptococci from closely related but distinct species within a single genus were compared (data not shown), there were often differences in the tDNA-PCR patterns (see also reference 16).

PCR products, each of about 160 bp, that were polymorphic in length among streptococcal groups A, B, and G (Fig. 1) were gel purified, reamplified, and cloned into pBSKII$^+$. Sequencing of these clones revealed that in each case they spanned part of tRNA^{Phe}, all of tRNA^{iMet}, and part of another unidentified tRNA. Selected parts of the sequences for these PCR products are presented in Fig. 2. These partial streptococcal tRNA^{Phe} and complete tRNA^{iMet} gene sequences were similar to those of *B. subtilis* (14), *Mycoplasma capricolum* (11), and *S. aureus* (17), all of which are members of the low G+C subdivision of the gram-positive phylum (22). However, the tRNA^{iMet} and tRNA^{Phe} genes are not known to be adjacent in *B. subtilis* (14), *M. capricolum* (11), or *S. aureus* (17). The nearest comparison is the order tRNA^{iMet}-tRNA^{Asp}-tRNA^{Phe}, which occurs in all three of these genera. Either the streptococcal sequences are from a new cluster or the gene order has diverged in these streptococci relative to the other related genera.

The tRNA^{iMet}-to-tRNA^{Phe} intergenic distance in our sample of streptococal species was 20 bp for group A, 2 bp for group B, and 18 bp for group G. The distances between the same tRNA genes in the published *B. subtilis* and *M. capricolum* clusters are 120 bp and 85 bp, respectively, including the intervening tRNA^{Asp} gene (11, 14). For staphylococci, the distance varies between 102 bp in *S. warneri* and 116 bp in *S. aureus* (17).

Amplification with primers designed for high-stringency PCR. The sequences for primers used for high-stringency PCR were based on sequences from the 15 bp between the 3' end of the consensus primer and the end of the tRNA^{iMet} gene and the 5' end of the tRNA^{Phe} gene, for which the complete sequence had been determined. These new primers were perfectly complementary to the sequences determined

FIG. 1. Fingerprints generated by tDNA-PCR in various *Streptococcus* species. Strains from four *Streptococcus* species were fingerprinted. PCR was performed by using the primers T5A and T3B with a 50°C annealing temperature and 1 ng of template. The products were resolved on a 5% acrylamide–50% urea gel and then autoradiographed. Each lane is the result of tDNA-PCR fingerprinting of a different strain. The arrow on the right indicates the region of the gel from which the products discussed in this paper were recovered. Lanes: 1, 2, and 3, three strains of group A streptococci; 4 and 5, two strains of group G streptococci; 6, a strain of group B streptococci; 7, *S. mutans* T8.

FIG. 3. PCR amplification with primers flanking the *Streptococcus* tRNA^{iMet}-tRNA^{Phe} intergenic spacer. PCR was performed with the primers SpytiMet3 and SpytPhe5 at 60°C with 1 ng of purified template DNA. The products were resolved on a 5% acrylamide–50% urea gel and then autoradiographed. In lane 15, a product of about 150 bp is not shown. Lanes: 1, *S. hominis*; 2 and 3, two strains of *S. warneri*; 4 and 5, two strains of *S. aureus*; 6 and 7, two strains of *S. haemolyticus*; 8, *S. cohnii*; 9 and 10, two strains of group A streptococci; 11 and 12, two strains of group B streptococci; 13 and 14, two strains of Group G streptococci; 15, *E. faecalis*; 16, *S. mutans* T8; 17, human.

in Fig. 2 and oriented in such a way that the intergenic region could be amplified. Out of 40 bases (2 × 20), these primers were noncomplementary to the *B. subtilis* tRNA^{iMet} and tRNA^{Phe} genes at one position, but were noncomplementary at five positions with the staphylococcal genes and nine positions with the mycoplasmal genes. Various high-stringency PCR protocols were experimented with to determine the maximum stringency that still permitted production of the expected PCR products from streptococcal genomes. The conditions chosen for the experiments shown below are described in Materials and Methods.

Fig. 3 shows the results of using the primers homologous to parts of tRNA^{iMet} and tRNA^{Phe} on various *Streptococcus*, *Staphylococcus*, and *Bacillus* species. tRNA-ILP products of the expected sizes are seen in group A, B, and G streptococci (71, 53, and 68 bp, respectively), whereas *S. mutans* gave a product of about 60 bp. Amplification with *Bacillus* species and *E. faecalis* (formerly *Streptococcus faecalis*) genomes gave PCR products of about 150 bp, indicating that, as expected, the tRNA^{iMet} and tRNA^{Phe} genes are homologous to the primers and are not as closely spaced in these species as they are in streptococci. Staphylococci gave no products.

Purified DNAs from a variety of other species were tested in the presence of an excess of human genomic DNA to determine whether these would yield tRNA-ILP products with the streptococcal tRNA^{iMet} and tRNA^{Phe} primers (Fig. 4). Although a few bacterial genera may give products longer than 150 bp, products shorter than 100 bp seem to be characteristic of members of the streptococci only. However, in order to determine the full array of species for which these primers can give a prominent and diagnostic product, as well as the size of the product, we are continuing our

	StrepiMet3		Spacer		StrepPhe5	
	AAGGTCGTAGGTTCAAATCC>>>>>>>			<<<<<<<<	GGCTCGGTAGCTCAGTTGGT	
group A *Strep.*	AAGGTCGCGGGTTCGAATCC	tgct CCCGCAA	**TACATAAGTATGTAGTTGTT**		GGCTCGGTAGCTCAGTTGGT	TAGAGC
group B *Strep.*	AAGGTCGTAGGTTCAAATCC	tgct CCCGCAA	**TA**		GGCTCGGTAGCTCAGTTGGT	TAGAGC
group G *Strep.*	AAGGTCGTAGGTTCAAATCC	tgct CCCGCAA	**TACATATAAATGTAGTT**		GGCTCGGTAGCTCAGTTGGT	TAGAGC
Staph. aureus	CGCGGGTTCGAATCC	GCCTCCCGCA	116 bp,	including tRNA^{Asp} gene	GGTTCAGTAGCTCAGTTGGT	
B. subtilis	AAGGTCGCAGGTTCAAATCC	TGCCCCCGCAACCA	120 bp,	including tRNA^{Asp} gene	GGCTCGGTAGCTCAGTTGGT	AGAGC
M. capricolum	GAGGTCGCAGGTTCGAGTCC	TGCCCCCGCAACCA	85 bp,	including tRNA^{Asp} gene	GGTCGTGTAGCTCAGTCGGT	AGAGC
		3' end of tRNA^{iMet}			5' end of tRNA^{Phe}	

FIG. 2. Sequences of the tRNA^{iMet}-tRNA^{Phe} intergenic spacer from groups A, B, and G streptococci. All *Streptococcus* (Strep) genes were sequenced from a minimum of two separate clones and in both strands. One region of a larger clone is shown. Intergenic spacers are in boldface type. Lowercase letters are uncertain sequences. The sequences are compared to those of the equivalent genes in *B. subtilis* (14), *M. capricolum* (11), and *S. aureus* (17). Underlined bases are differences between the primers and tRNA gene sequences. The complement of the primer StrepPhe5 is shown.

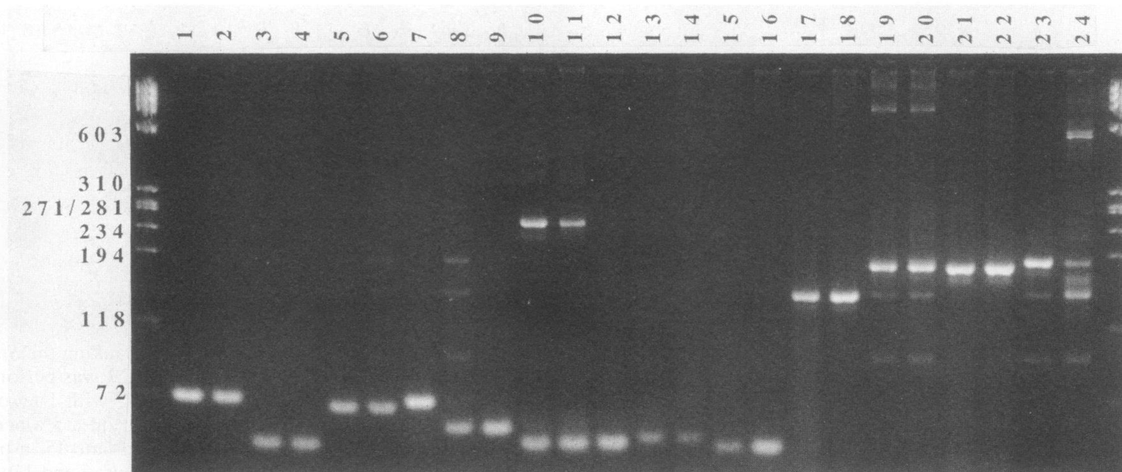

FIG. 4. PCR survey of various genomes with primers designed for the tRNA^{iMet}-tRNA^{Phe} intergenic spacer of streptococci. PCR was performed by using the primers SpytiMet3 and SpytPhe5 at 60°C with 1 ng of template DNA and 1,000 ng of human DNA. The DNAs were resolved on a 1.5% agarose Tris-borate-EDTA gel. Molecular weight markers are PhiX174 DNA digested with *Hae*III. Lanes: 1, *S. pyogenes* 52RS15; 2, *S. pyogenes* Ti/195/2; 3, *Streptococcus* group B B16501; 4, *Streptococcus* group B 501-1b; 5, *Streptococcus* group G 1/E9; 6, *Streptococcus* group G 1/B1/A2; 7, *S. pyogenes* ATCC 14289; 8, *S. mutans* GS5; 9, *S. mutans* ATCC 35668; 10, *S. pneumoniae* S. Lacks; 11, *Streptococcus mitis* ATCC 9811; 12, *Streptococcus salivarius* ATCC 13419; 13, *S. sanguis* ATCC 10556; 14, unidentified *Streptococcus* clinical sample; 15, *Streptococcus bovis* ATCC 35034; 16, BHSB no. 114 2613A; 17, *Streptococcus faecium* 4763A; 18, *S. faecium* 1594; 19, *E. faecalis* JH2-2; 20, *E. faecalis* OG1X; 21, *E. faecalis* 3537B; 22, *E. faecalis* ATCC 19433; 23, *B. globigii* G. A. Wilson; 24, *Bacillus amyloliquefaciens* H F. E. Young.

systematic analysis of *Streptococcus* species and related genera. In particular, the various Lancefield groups have been divided into species, and it remains to be seen whether these can all be distinguished by tDNA-ILPs. Although the primers that we have developed can distinguish streptococci from related genera and can distinguish between some *Streptococcus* species, it seems likely that at least one more pair of primers spanning a different tRNA intergenic spacer will be necessary to distinguish all *Streptococcus* species from each other.

In the experiments presented here, only one product was generated in most species by using the tRNA^{iMet} and tRNA^{Phe} primers. For some pairs of PCR primers, one can expect that there will be two (or more) products, because tRNA genes are often duplicated in the genome and the organization of the tRNA genes in the two clusters can be similar. This phenomenon, when it occurs, can be of help in distinguishing species, because the lengths of the two products both vary between species and do so independently (17). An example of this phenomenon occurs for *Streptococcus pneumoniae* (Fig. 4, lane 10).

DISCUSSION

PCR of tDNA-ILPs. We wished to test a PCR method for identifying species- or strain-specific length polymorphisms. The streptococci were chosen as a model because they represent a diverse set of species, many of which are pathogenic to humans (5).

Primers to be used in high-stringency PCR were derived by a two-step process. First, consensus tRNA gene primers were designed that can be used, under low-stringency PCR conditions, to amplify by PCR a set of tRNA intergenic regions, some of which vary in length between related species (16). These products were generated by low-stringency tDNA-PCR, so it was possible that products of similar sizes derived from different species were not homologs. However, we hypothesized that because tRNA gene sequences and their organization are unlikely to differ very much between species within a genus, products in a particular portion of the gel are likely to be from homologous regions when closely related species are compared. This was indeed the case for streptococci, since cloning and sequencing showed that the variation in tDNA-PCR fingerprints observed between streptococcal species is due to length polymorphisms in the intergenic spacers.

Primers with perfect homology to the sequences determined for tRNA genes that flanked tDNA-PCR length polymorphisms were used to amplify the corresponding polymorphic intergenic spacers from several species. These new primers, unlike the consensus primers used to initially identify the polymorphisms, can be used at high stringency and in the presence of DNAs from other sources, such as human DNA in medical samples. We refer to the differences in the lengths of the PCR products as tRNA-ILPs. The size of the PCR product helps to distinguish between species in the genus *Streptococcus*. Because this strategy can be applied to any genus, many groups of human pathogens could be detected and categorized into species by using only a small number of primers.

Although streptococci were chosen in this study as a model for a general method, the tRNA-ILP primers developed here may be useful for diagnosis, despite the expertise already accumulated for antigen-based detection and categorization for some strains, particularly Lancefield group A. Although such kits often have a very high specificity, sensitivity is usually not as high (4, 6, 7, 9, 10). A few cases of infection are likely to be caused by strains of streptococci that are not detected by standard kits. PCR of tRNA gene spacers may be more sensitive and reliable than immunological methods, while also identifying the strain responsible for the occasional infection by a rare group of streptococci. Furthermore, the detection of tRNA-ILPs is relatively fast because the products can be designed to be short, allowing

the cycling parameters to be rapid. Contaminants have difficulty competing against a short substrate. The products run rapidly on a gel, and a difference in size of a few base pairs results in an easily detectable change in mobility. In a clinical setting, the use of the dUTP-*ung* system to minimize cross-contamination (Perkin-Elmer Cetus) should make a routine test quite reliable.

tDNA-PCR and tDNA-ILPs in other genera. If pure cultures are available, tDNA-PCR with consensus primers (16) can be used for species identification and arbitrarily primed PCR can be used for intraspecies comparisons (15, 18–20). However, for uncultured materials, impure cultures, and detection of very low levels of the species of interest, the tRNA-ILP method is preferable.

The consensus tRNA gene primers developed for tDNA-PCR (18) generate fingerprints in many species of bacteria. In addition to the experiments shown here, we have used these consensus primers to create fingerprints that show interspecific polymorphisms in *Staphylococcus*, *Borrelia*, *Rickettsia*, and *Mycobacterium* species (17a). Polymorphisms in these fingerprints identify spacers that are good candidates for sequencing and production of primers for high-stringency PCR by the tRNA-ILP method. In this manner, primers for high-stringency PCR that generate polymorphic DNA products may be developed for any group of bacteria and perhaps even for lower eukaryotes of medical importance, such as *Pneumocystis carinii* and *Aspergillus* and *Candida* species that cause fungal infections, for example.

Other intergenic spacer polymorphisms. In principle, one could distinguish between species on the basis of length polymorphisms in any region of the genome. However, PCR primers for the detection of these polymorphisms are best located in relatively conserved regions because noncoding sequences differ substantially, even in the genomes of closely related species. Primers could be located in very conserved protein-coding regions to amplify intergenic noncoding regions. However, whether these primers detected length polymorphisms would be a matter of trial and error. In contrast, tRNA intergenic length polymorphisms were chosen as our diagnostic tool because species- or strain-specific length polymorphisms could be identified by using the simple consensus tRNA primer strategy. These polymorphisms could then be used to yield a DNA sequence that would be the basis for the development of primers for high-stringency PCR, thereby distinguishing a large group of related species.

Species- or strain-specific ILPs could be based on rRNA genes, the sequences and organization of which are well conserved in bacteria. We have demonstrated the principle of using primers to amplify the intergenic regions between 16S and 23S and between 23S and 5S as a diagnostic tool (17a). The lengths of these spacers are variable between species and could be used to distinguish them. Such PCR products are termed rRNA-ILPs. Given the rapidly accumulating sequence data for rRNA genes, the production of genus-specific primers may often not require an rRNA consensus fingerprint as a first step. This is also true for the large amounts of information about tRNA gene sequences and organization that have become available, such as for *Mycoplasma* species (11).

Intergenic length polymorphisms based on tRNA-ILPs, rRNA-ILPs, and perhaps protein-ILPs could be developed for any genus. These represent a potentially valuable source of tools for rapid species or strain identification independent of phenotypic characteristics.

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