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Development of a Highly Specific Assay for Rapid Identification of Pathogenic Strains of *Yersinia enterocolitica* Based on PCR Amplification of the *Yersinia* Heat-Stable Enterotoxin Gene (*yst*)

ASHRAF IBRAHIM,[1]* WERNER LIESACK,[2] MANSEL W. GRIFFITHS,[1] AND ROY M. ROBINS-BROWNE[3]

Applied Microbial Genetics Division, Department of Food Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada[1]; Max-Planck-Institut für Terrestrische Mikrobiologie, D-35043 Marburg, Germany[2]; and Microbiological Research Unit, Department of Microbiology and Infectious Diseases, Royal Children's Hospital, and Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3052, Australia[3]

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The chromosomal gene *yst*, which encodes a heat-stable enterotoxin of *Yersinia enterocolitica*, is a useful diagnostic marker because it occurs only in invasive strains of this species. A homologous gene also occurs in some strains of *Yersinia kristensenii*. Sequence analysis of the *yst* genes from two different strains of *Y. enterocolitica* and from *Y. kristensenii* revealed a substantial number of mismatches at the 3′ ends of the *yst* genes of the so-called American and European biotypes of *Y. enterocolitica*. Moreover, several mismatches and a deletion of 5 codons were found in the *yst* of *Y. kristensenii*. These findings were used to develop a PCR-based assay for *yst* of *Y. enterocolitica* which yielded a detectable product in as little as 50 min. The assay was 100% specific in terms of its ability to identify potentially pathogenic strains of *Y. enterocolitica* regardless of biotype or serotype. The PCR yielded an amplicon that was visible on agarose gel electrophoresis from as few as 100 CFU, or 10 CFU when the PCR was combined with dot blot hybridization with a digoxigenin-labeled oligonucleotide probe that corresponded to an internal sequence of *yst*. These results establish the value of the *yst* gene as a target for the identification of pathogenic bioserotypes of *Y. enterocolitica* and the usefulness of PCR for this purpose.

Yersinia enterocolitica is an enteric pathogen which causes acute gastroenteritis, enterocolitis, and mesenteric adenitis, as well as a variety of extraintestinal disorders (2, 7). Only a few clones among the 6 biotypes and more than 60 serotypes of *Y. enterocolitica* described to date are considered to be primary pathogens (2, 7). Biotyping permits the division of virulent clones into two broad groups, commonly referred to as the American and European varieties. American strains belong to biotype 1B and include serotypes O:8, O:13a,13b, O:20, and O:21, whereas European strains belong to biotypes 2 through 5 and include serotypes O:3, O:5,27, and O:9.

Virulence in *Y. enterocolitica* results from a complex interplay between a series of plasmid-borne and chromosomal genes (1, 7). The latter include *yst*, the chromosomal gene encoding a low-molecular-weight, heat-stable enterotoxin which belongs to a family of structurally and functionally related enterotoxins produced by several species of diarrheagenic bacteria (9–11). Although *yst* is confined to pathogenic bioserotypes of *Y. enterocolitica* and hence is a useful marker of potential virulence, a homologous gene is found in some isolates of *Yersinia kristensenii* (3, 5), despite the fact that *Y. kristensenii* is seldom associated with disease (8).

We reported previously that a primer set designed to amplify *yst* from *Y. enterocolitica* by PCR generated amplicons with two different electrophoretic profiles depending on whether the target strain was of the American or the European variety (4). In addition, the PCR yielded a product from *Y. kristensenii* which was not recognized by a 20-mer oligonucleotide DNA probe based on the nucleotide sequence of the *yst* of a European strain of *Y. enterocolitica* (6). Taken together, these observations pointed to possible heterogeneity of the *yst* gene in the American and European varieties of *Y. enterocolitica* and in *Y. kristensenii*.

In the present study, we investigated these findings further by performing PCR on *Y. kristensenii* 490 (serotype O:12,25) with primers Pr1a and Pr1b, which are based on the sequence of *yst* from *Y. enterocolitica* W1024 (serotype O:9; European type) (Fig. 1) (3, 5). For the PCR, bacteria were suspended in 100 μl of distilled water to which was added 5 μl of 20% sodium dodecyl sulfate. The mixture was incubated at 60°C for 10 min, after which the clear cell lysate was purified by using a GeneClean kit (BIO 101, La Jolla, Calif.) as described previously (4). The reaction was carried out in a final volume of 50 μl containing 100 mM each deoxynucleoside triphosphate, 10 pmol of each primer, 1× Taq buffer (Boehringer GmbH, Mannheim, Germany), and 5 μl of template DNA. Tubes were heated to 80°C, after which 1 U of *Taq* polymerase (Boehringer) and then 50 μl of mineral oil were added. PCR was performed for 30 cycles of 1 min at 94°C (for denaturation of the template DNA), 1 min at 52°C (for annealing), and 1 min at 72°C (for extension). The assay concluded with a 5-min extension period at 72°C. The product of this reaction was recovered from the agarose gel, purified by using a GeneClean kit, and resuspended in 10 μl of distilled water. Direct sequencing was carried out by using the ds DNA Cycle Sequencing System (Bethesda Research Laboratories, Gaithersburg, Bethesda, Md.) with the three sequencing primers described previously (6).

Analysis of the *yst* sequences from representative American

* Corresponding author. Present address: Division of Microbiology, National Defense Research Institute, S901-82 Umeå, Sweden. Phone: 46 90106818. Fax: 46 90106806. E-mail: ibrahim@ume.foa.se.

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                          Primer: Pr1a                    Primer: Pr2a
Strain W1024   ATG AAA AAG ATA GTT TTT GTT CTT GTG TTA ATG CTG TCT TCA TTT GGA GCA TTC GGC CAA GAA ACA GTT TCA TTT   75
Strain 8081    ATG AAA AAA ATA GTT TTT GTT CTT GTG TTA ATG CTG TCT TCA TTT GGA GCA TTC GGC CAA GAA ACA GTT TCA TTT
Strain 490     ATG AAA AAA ATA GTT TTT GTT CTT GTG TTA ATG CTG TCT TCA TTT AGA GCA TTC GGC CAA AGG ACA GCT TCG AGG   75

                                                                                          Probe
Strain W1024   CAG TTC AGT GAT GCA TTA TCG ACA CCA ATA ACC GCT GAG GTA TAC AAG CAA GCT TGT GAT CCT CCG TCG CCA CCA  150
Strain 8081    CAG TTC AGT GAT GCA TTA TCG ACA CCA ATA ACC GCT GAG GTA TAC AAG CAA GCT TGT GAT CCT CCG CTG CCA CCA
Strain 490     CAG TTT GGT GAT GCA TTC TCG ACA CCT ATC GCC GCT GAG GTA AAC AAA AAA GCT TGC GAT ACT GAA TTG CCG CCC  150

                          Primer: Pr2c                                          Primer: Pr1b
Strain W1024   CCC GAA GTC AGT AGT GAT TGG GAT TGC TGT GAT GTA TGT TGC AAT CCC GCG TGT GCT GGC TGC TAG  216
Strain 8081    CCC GAA GTC AGT AGT GAT TGG GAT TGC TGC GAT GTA TGT TGC AAT CCT GCC TGT GCG GGT TGC TAG
Strain 490     TCT --- --- --- --- GAT TGG --- TGC TGT GAG GTA TGC TGC AAT CCC GCG TGT GCT GGC TGC TAG  201
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FIG. 1. Comparison of the nucleotide sequences of the *yst* genes from *Y. enterocolitica* W1024 (serotype O:9; European type) (3) and 8081 (serotype O:8; American type) and from *Y. kristensenii* 490 (serotype O:12,25). Nucleotide sequences of strain 8081 or 490 that differ from that of strain W1024 are underlined. Dashes indicate deletions. Sequences corresponding to the primers used for the PCR assays reported in this study are shown in italics and overscored. A 20-mer oligonucleotide probe used to detect and/or confirm the identity of PCR products obtained from *Y. enterocolitica* is shown in boldface type and overscored.

and European strains of *Y. enterocolitica* revealed four mismatches at the 3′ end, which could have accounted for the failure of primer set Pr1a-Pr1b to yield the expected 208-bp amplicon from American strains (Fig. 1). In addition, the sequence of the *yst* gene of *Y. kristensenii* revealed mismatches in the sequence corresponding to the oligonucleotide probe (6), as well as a 15-bp deletion corresponding to nucleotides 220 to 231 and 238 to 240 of *yst* from *Y. enterocolitica* (Fig. 1). Interestingly, the deletion did not affect the highly conserved carboxyl terminus of the mature toxin, which evidently is required for the function of enterotoxins of this type (Fig. 2) (11). The difference in the sizes of the amplicons obtained by PCR amplification of DNA from *Y. enterocolitica* and *Y. kristensenii* was not evident when the products of the PCR were analyzed by electrophoresis in 1.3% agarose gels but became apparent when the agarose concentration was increased to 2% (Fig. 3).

The sequence data of the three *yst* genes depicted in Fig. 1 were used to design new PCR primers, Pr2a and Pr2c. The latter was designed to target a relatively conserved region of *yst* of *Y. enterocolitica* encompassing nucleotides 154 to 174, which corresponded to the deleted region in *Y. kristensenii*. These primers were used in a PCR similar to that described above except that each cycle consisted of 30 s at 94°C (for denaturation of the template DNA) and 30 s at 60°C (for annealing and extension). The PCR using primer set Pr2a-Pr2c was highly specific for amplification of the target sequence, giving rise to a 145-bp PCR product from all pathogenic strains of *Y. enterocolitica* regardless of bioserotype (data not shown). Importantly, all of 20 nonpathogenic isolates of *Y. enterocolitica* and 41 other *Yersinia* species (including 9 strains of *Y. kristensenii* of different origin) did not yield any amplification products in the PCR which employed these primers. The specificity of primer set Pr2a-Pr2c was confirmed by performing the PCR with 36 strains of other enterotoxigenic bacterial species, including several which produce homologs of Yst, and demonstrating that none of them gave a PCR product. In addition, a search of the EMBL database did not reveal any other sequence with significant DNA homology to primer set Pr2a-Pr2c.

The sensitivity of the PCR based on primers Pr2a and Pr2c was determined by examining serial 10-fold dilutions of an overnight culture of a serotype O:3 (European) strain of *Y. enterocolitica*, which contained 10^5, 10^4, 10^3, 10^2, or 10 CFU. After DNA had been extracted and the PCR was performed as described above, PCR amplicons from each reaction were precipitated, spotted on nylon membranes, and fixed by exposure to UV radiation for 5 min. Hybridization was carried out with a digoxigenin-labeled internal probe based on an internal region of *yst* (Fig. 1) as described previously (5). Bound probe was detected by using Lumigen PPD (Boehringer) as the substrate and was visualized by using a Bioimage Quantifier (BIQ, Cambridge, United Kingdom). The results showed that when gel electrophoresis was used as the detection method, the detection limit of the PCR was 10^2. When dot blot hybridization with the digoxigenin-labeled oligonucleotide probe was

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Y. enterocolitica O:9 (W1024) [a]   Q A C D P P S P P P E V S S D W D C C D V C C N P A C A G C
Y. enterocolitica O:8 (8081) [b]    Q A C D P P L P P P E V S S D W D C C D V C C N P A C A G C
Y. kristensenii [b]                       K A C D T E L P P S D W C C E V C C N P A C A G C
E. coli STa (porcine type) [c]                          N T F Y C C E L C C N P A C A G C Y
E. coli STa (human type) [c]                N S S N Y C C E L C C N P A C T G C Y
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FIG. 2. Alignment of the amino acid sequences of mature heat-stable enterotoxins of *Y. enterocolitica*, *Y. kristensenii*, and enterotoxigenic *Escherichia coli* of porcine and human origin. The amino acid residues in boxes are common to all five peptides. Footnotes: a, data are from reference 3; b, data are from this study; c, data are from reference 10.

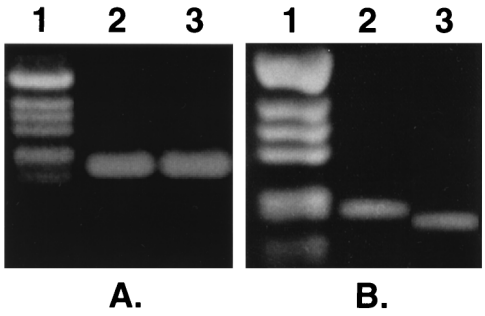

FIG. 3. Electrophoresis patterns, on 2% (A) and 1.3% (B) agarose gels, of amplification products derived from *yst*-specific PCR of *Y. kristensenii* and *Y. enterocolitica* with primer set Pr1a-Pr1b (Fig. 1). Lanes: 1, 1-kb ladder (Bethesda Research Laboratories); 2, *Y. kristensenii*; 3, *Y. enterocolitica*, serotype O:3 (European type).

used as the detection method, an amplicon was readily detected in the 10-CFU sample (data not shown).

Because pathogenic and nonpathogenic varieties of *Y. enterocolitica* may be found in similar types of samples submitted to diagnostic laboratories, there is a need to be able to distinguish the two forms from each other. PCR based on the chromosomal *yst* gene, which is absent from nonpathogenic bioserotypes of *Y. enterocolitica*, permits the rapid identification of clinically significant isolates from food, water, or clinical material (6). We previously reported that a PCR assay to detect pathogenic strains of *Y. enterocolitica* based on amplification of *yst* gave different results with *Y. enterocolitica* strains of American and European biotypes and with some strains of *Y. kristensenii*, suggesting that the nucleotide sequences of the *yst* genes in these three groups of bacteria may differ (4). Determination of the *yst* sequences of representative strains of these bacteria revealed that these discrepancies are due to (i) differences in the sequences of the gene in American and European strains of *Y. enterocolitica* in the region of a 3′ primer (Pr1b) and (ii) differences in the *yst* genes of *Y. enterocolitica* and *Y. kristensenii* in the region recognized by a diagnostic oligonucleotide probe (Fig. 1).

The sequence data derived during this study were used to design novel primers, Pr2a and Pr2c, for a specific PCR which yielded a consistent product regardless of the bioserotype of the target strain. Although we determined the nucleotide sequence of only one representative strain from each group, the results of the PCR obtained with 85 strains of *Yersinia* species, including all pathogenic bioserotypes of *Y. enterocolitica* (16 American and 8 European strains) and 9 strains of *Y. kristensenii*, suggested that these changes are conserved within each category of bacteria. The PCR using primer set Pr2a-Pr2c was 100% specific and highly sensitive, allowing the detection of 100 CFU, or even 10 CFU when combined with dot blot hybridization with an oligonucleotide probe directed against an internal sequence of the PCR product. The original PCR based on primers Pr1a and Pr1b remains useful, however, because it provides some information concerning the biotypes of the strains as well as potential virulence and also because it permits amplification of *yst* from *Y. kristensenii*, should this be required.

The PCR based on primers Pr2a and Pr2c involved one-step bacterial lysis with sodium dodecyl sulfate and subsequent rapid purification of DNA with a GeneClean kit. No further extraction or precipitation steps were required, thus simplifying the procedure and minimizing the risk of DNA cross-contamination. The conventional PCR thermal profile involving three-stage cycles of denaturation, annealing, and extension was found to be unnecessary. Indeed, the inclusion of separate annealing and extension steps tended to result in nonspecific amplification products (data not shown). By carefully monitoring the thermal cycling profile, we showed that 30 s at 94°C is sufficient to denature double-stranded DNA and that longer denaturation periods may affect the integrity of the PCR products.

In conclusion, we describe a novel PCR for the detection of potentially pathogenic strains of *Y. enterocolitica* based on amplification of the *yst* gene. The PCR took into account sequence variation in the *yst* genes of the American and European bioserotypes of *Y. enterocolitica* as well as variation in the genes of *Y. enterocolitica* and *Y. kristensenii*. The assay is highly specific and sensitive and can provide a result in as little as 50 min.

Nucleotide sequence accession numbers. The *yst* sequence of *Y. kristensenii* 490 and that of *Y. enterocolitica* 8081 (serotype O:8 [American]), reported previously (4), have been deposited in the EMBL database under accession numbers X65999 and X69218, respectively.

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