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Polymerase Chain Reaction-Gene Probe Detection System Specific for Pathogenic Strains of *Yersinia enterocolitica*

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The polymerase chain reaction technique was used to develop a rapid diagnostic assay for detection of pathogenic *Yersinia enterocolitica* strains. The assay targeted a stretch of 163 bp of the *yst* gene and could be applied to both pure cultures and crude DNA extracted from feces. The defined primer pair amplified the targeted sequence from only pathogenic strains and fecal samples seeded with the serotype O:3 strain of *Y. enterocolitica*, whereas neither nonpathogenic strains nor normal stools yielded any amplified fragments. Of the other *Yersinia* species and non-*Yersinia* species tested, only two strains of *Y. kristensenii* yielded the same amplified product. A 20-mer oligonucleotide probe specifically hybridized within the amplified *yst* fragment of *Y. enterocolitica* but did not hybridize with the amplified *yst* fragment of *Y. kristensenii* by Southern and dot blot hybridizations. This confirms the reliability of this diagnostic assay in both clinical and epidemiological studies. The availability of the extracted DNA for the polymerase chain reaction was checked by simultaneous amplification of a part of the 16S rDNA and the *yst* gene. The entire diagnostic assay, including a simplified technique for DNA extraction, the amplification process, and gel electrophoresis, could be completed within 1 working day, which is better than the time required for the time-consuming traditional techniques used in clinical laboratories.

Yersinia enterocolitica is an enteric pathogen which causes gastrointestinal disorders with a wide range of clinical manifestations, from mild diarrhea to mesenteric lymphadenitis (5). Complicated infections manifested by arthritis (1), erythema nodosum (28), and septicemia (2) are not uncommon. In addition, fatal cases of yersiniosis caused by transfusion of infected blood have been reported in several countries (4, 26). The species comprises a heterogenous group of organisms with more than 50 serovars and several biotypes (23). However, the pathogenic serovars commonly associated with human yersiniosis are limited to O:1,3; O:3; O:9; and O:5,27 (known as European strains) and O:8; O:13a,13b: O:20; O:21; O:18; and O:4,32 (known as American strains). Phenotypic differentiation between pathogenic and nonpathogenic strains of *Y. enterocolitica* necessitates a battery of biochemical and serological tests which have been reported to provide inconsistent results and to be time-consuming (18). Genotypic markers for pathogenicity have been attributed to both chromosomal and plasmid loci (8, 16, 29). Consequently, different DNA probes were developed to rapidly detect the organism in environmental samples (10, 11, 17) and to epidemiologically differentiate between virulent and avirulent (6, 19), strains. The diagnostic value of the probes that target plasmid-encoded sequences, however, has been questioned on the following grounds: (i) the accidental loss of the plasmid during isolation would give false-negative results, and (ii) the target sequences of these probes are not exclusive for *Y. enterocolitica* but are highly conserved in the DNAs of *Y. pseudotuberculosis* and *Y. pestis* as well (25). The need for a reliable and rapid diagnostic assay to detect pathogenic strains of *Y. enterocolitica* specifically is clinically and epidemiologically important. In a previous study (9) we reported the generation of a vector-free digoxigenin-dUTP-labeled probe that targeted the chromosomally encoded *yst* gene (*Y. enterocolitica* heat-stable enterotoxin) by using DNA-DNA colony hybridization. However, the positive hybridization of the *yst* probe with three strains of *Y. kristensenii* was a limiting factor from an epidemiological point of view. In addition, time- and cost-effective diagnostic tests are crucial for validating any diagnostic assay in clinical laboratories. We report here a polymerase chain reaction (PCR)-mediated detection system that is exclusive for pathogenic strains of *Y. enterocolitica* and its applicability to pure cultures and clinical specimens.

MATERIALS AND METHODS

Bacterial strains. (i) Reference strains. The strains of *Y. enterocolitica* and related species used in this study were chosen to include representatives of almost all known pathogenic biotypes and serotypes (Table 1) from a variety of sources in North America, Europe, Asia, and Australia. Details of these strains were mentioned in a previous report (9). The other bacterial strains tested in this study are listed in Table 2.

(ii) Hospital strains. Eleven strains of *Y. enterocolitica* (five from Princess Alexandra Hospital, Brisbane, Queensland, Australia, and six from Royal Brisbane Hospital), which were identified to the species level by conventional techniques, were forwarded to our laboratory for confirmation of the pathogenicity by positive identification of the *yst* gene.

Fecal samples. To evaluate the reliability of the diagnostic assay in feces, three different sets of stool specimens were examined during the course of our experiment: (i) normal stool specimens (30 samples), (ii) normal stool specimens inoculated with approximately 10^4 CFU of an overnight culture of a serotype O:3 strain of *Y. enterocolitica* (5 samples), and (iii) diarrheal stools obtained from Princess

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TABLE 1. *Y. enterocolitica* and related species checked for the *yst* gene

Species	Serotype	Total no. of strains tested	No. of strains *yst*+[a]
Y. enterocolitica (pathogenic)	1,3	2	2
	2a,3	1	1
	3	21	21
	4,32	1	1
	5,27	5	5
	8	7	7
	9	7	7
	13a,13b	3	3
	20	3	3
	21	3	3
Y. enterocolitica (nonpathogenic)	4,33	5	0
	5	11	0
	7,8	3	0
	7,8,9	2	0
	7,13,19	1	0
	10,34	2	0
	21[b]	3	0
	22	1	0
	34	2	0
	NT[c]	2	0
Y. pseudotuberculosis	ND[d]	2	0
Y. kristensenii	11	1	0
	12,25; 12,26	2	0
	16	1	0
	52	1	0
Y. frederiksenii	3[b]; 58–73; 16–16, 29; NT	4	0
Y. intermedia	3; 4,33; 17; 17,48	4	0
Y. ruckeri	ND[d]	2	0

[a] Strains positive for both PCR and hybridization with the internal probe.
[b] Atypical nonpathogenic strains.
[c] NT, nontypeable.
[d] ND, not determined.

TABLE 2. Bacterial species other than yersiniae included in the diagnostic assay

Bacterial species	Total no. of strains tested[a]
Escherichia coli[b]	
ETEC (LT)	5
ETEC (ST)	2
Nonpathogenic	2
Salmonella enteritidis	3
Salmonella typhimurium	2
Salmonella dublin	1
Salmonella berta	1
Salmonella javiana	1
Salmonella eastbourne	1
Shigella dysenteriae	2
Shigella sonnei	1
Klebsiella pneumoniae	1
Aeromonas hydrophila	2
Aeromonas sobria	2
Vibrio cholerae	1
Campylobacter jejuni	1

[a] None of the strain were *yst*+, which indicates the presence of a PCR product.
[b] ETEC (LT), heat-labile enterotoxigenic *Escherichia coli*; ETEC (ST), heat-stable enterotoxigenic *Escherichia coli*.

Alexandra Hospital and Sullivan & Nicholaides Clinical Laboratories, Brisbane (35 samples). Both laboratories routinely receive samples from different localities in the state of Queensland and screen diarrheal stools for *Y. enterocolitica* by the same conventional techniques, i.e., selective isolation on cefsulodin-irgasan-novobiocin agar followed by biochemical identification of presumptive colonies.

Preparation of oligonucleotide primers and internal probe. Oligonucleotides were synthesized by using a 391 DNA assembler (Applied Biosystems, Burwood, Victoria, Australia) and were purified as recommended by the manufacturer. The *yst* primers and the internal probe were defined to be homologous to a region of the *yst* gene by using the sequence reported by Delor et al. (6) (Fig. 1). The internal probe was 5′ labeled with [γ-^{32}P]ATP (Bresatech, Adelaide, South Australia, Australia) by using T4 polynucleotide kinase as described by Krupp (13). The labeled probe was purified by using a Sephadex G-50 spin column (21). Nonradioactive labeling was carried out with digoxigenin-dUTP (Boehringer, Mannheim, Germany) by tailing with terminal transferase; this was followed by purification by selective precipitation with lithium chloride and absolute ethanol (24). The availability of DNA for amplification in the samples was tested by multiplex PCR. Two universal 16S rDNA primers, at positions 9 to 26 (14) and 343 to 357 (International Union of Biochemistry nomenclature of *Escherichia coli* [27]), that amplified approximately 350 bp of the 16S rDNA simultaneously with the *yst* gene were used. The sequences of all primers used in our investigation are given in Table 3.

Preparation of bacterial DNA. (i) DNA preparation from pure cultures. Test bacteria were grown overnight in brain heart infusion broth (Difco, Detroit, Mich.) at 28°C. Cells were pelleted from 1 ml of broth and were resuspended in 100 μl of distilled water. Five microliters of sodium dodecyl sulfate (SDS) (20%) was added, and the tubes were incubated for 10 min at 60°C. The clear cell lysate that was obtained was purified by using the Isogene Kit (Perkin-Elmer Cetus, Melbourne, Victoria, Australia), with a slight modification. Briefly, 200 μl of sodium iodide was added to the lysate, and the tubes were incubated in ice water for 5 min. One microliter of DNA binder (instead of 10 μl, as recommended by the manufacturer) was added, and the solution was mixed for 10 min. Bound DNA was precipitated by centrifugation for 1 min and was washed twice with the washing buffer; this was followed by complete aspiration of the washing solution. DNA was then eluted in a final volume of 60 μl (three times, 20 μl) of distilled water and was directly subjected to amplification as described below.

(ii) DNA preparation from fecal samples. Approximately 200 mg of each sample was suspended in 1 ml of phosphate-buffered saline (pH 7.4). Coarse particles were removed by low-speed centrifugation, and bacterial cells in the supernatant were pelleted by centrifugation for 1 min in an Eppendorf microcentrifuge. The pellet was washed twice in 1 ml of

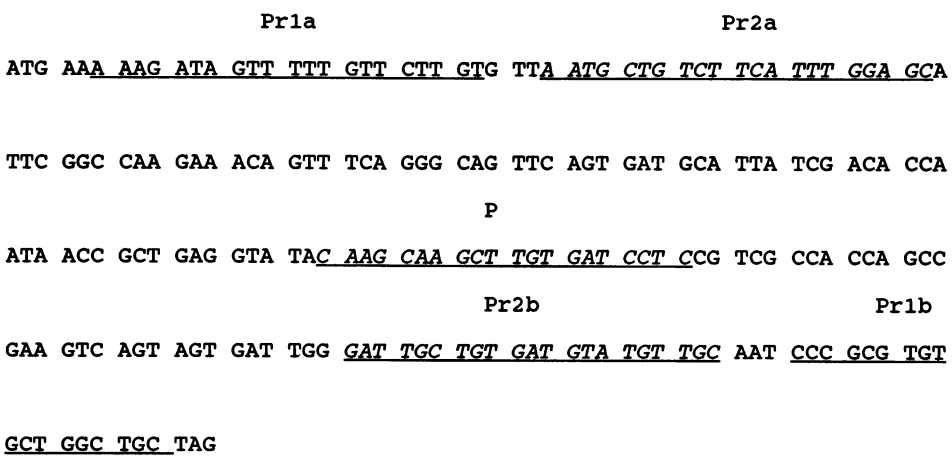

FIG. 1. Nucleotide sequence of the *yst* gene as published by Delor et al. (6) and the position of the primer sets and the internal probe that were used.

TE buffer (10 mM Tris-HCl plus 1 mM EDTA) (pH 8.0) and was resuspended in 200 μl of the same buffer. The cells were lysed by adding 10 μl of SDS (20%) and subsequent incubation for 30 min at 60°C. The cell lysate was purified as described above, except that the DNA binder was increased to 5 μl and the bound DNA was washed three times with washing buffer before being eluted with distilled water.

PCR-mediated DNA amplification. DNA was amplified by PCR (20) by using 1 to 5 μl of extracted DNA, 0.2 μg of each of the *yst* primers, and 0.4 μg of each of the 16S rDNA primers (Table 3). PCR was performed for 35 cycles for 1 min at 93°C (denaturation), 2 min at 60°C (annealing of primers to single-stranded DNA), and 20 s at 72°C (elongation). Ten microliters of the amplified products (the partial *yst* and 16S rRNA genes) were visualized by electrophoresis by using a 1.3% agarose gel.

Detection of the *yst* gene with the internal probe. (i) Use of the [γ-^{32}P]ATP-labeled probe. DNA was transferred from agarose gels to nylon membrane filters (Hybond N; Amersham International, Amersham, England) by Southern blotting (21). Filters were air dried, wrapped in plastic, and exposed to UV light (302 nm) for 5 min to fix the single-stranded DNA to the filter matrix. Hybridization proceeded for 2 h at 50°C by using a buffer containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 0.1% SDS, 1 mM EDTA, 1× Denhardt solution (7), 250 μg of calf thymus DNA per ml, and the denatured labeled probe. Filters were then washed twice in 2× SSC–0.1% SDS for 15 min and once in 2× SSC for 10 min at the hybridization temperature, dried, and exposed to X-ray film at −70°C for 4 h.

(ii) Use of the digoxigenin-dUTP-labeled probe. PCR products were precipitated with an equal volume of 4 M ammonium acetate and a double volume of isopropanol, washed once with 70% ethanol, and resuspended in 10 μl of distilled water. One microliter of each sample was spotted onto nylon membranes (see above). Denaturation of the DNA, hybridization, and visualization were performed as mentioned previously (9), except that the prehybridization step was omitted and hybridization was done for only 1 h.

Sensitivity of the diagnostic assay. An overnight culture of a *Y. enterocolitica* serotype O:3 strain containing approximately 10^4 CFU was serially diluted, and normal stool specimens were seeded with each dilution. DNA was extracted as described above. To ensure maximum recovery of DNA, 10 μl of DNA binder was used. Five microliters of the purified DNA was subjected to amplification by PCR.

TABLE 3. Sequence of the oligonucleotide primer sets and the internal probe targeting the *yst* or 16S rRNA genes

Primer[a]	Sequence[b]	T_m (°C)[c]
yst-Pr2a	5′-^{30}AATGCTGTCTTCATTTGGAGC^{50}-3′	60
yst-Pr2b	5′-^{192}GCAACATACATCACAGCAATC^{172}-3′	60
yst-P	5′-^{120}CAAGCAAGCTTGTGATCCTC^{139}-3′	60
16S rDNA-Pr2a	5′-^{9}GAGUUUGAUCCUGGCUCA^{26}-3′	54
16S rDNA-Pr2b	5′-^{357}CTGCTGCCTCCCGTA^{343}-3′	50

[a] Pr, primer; P, probe.
[b] Superscript numbers indicate the positions of the primers within the *yst* reading frame (6) and 16S rDNA genes (International Union of Biochemistry nomenclature of *E. coli*), respectively.
[c] T_m, melting temperature determined as described by Mason and Williams (15).

RESULTS

Preparation and purification of template DNA. All bacterial strains tested in this investigation were easily lysed with SDS alone. The availability of the extracted DNA for PCR was checked by simultaneous amplification of the *yst* gene and the 16S rDNA fragment. DNA from pathogenic strains of *Y. enterocolitica* gave rise to two amplified products, while nonpathogenic strains yielded only a single rDNA fragment (Fig. 2A). In coamplification with the *yst* gene, the 16S rDNA fragment was amplified less efficiently than it was in the absence of the *yst* target.

Amplification of the *yst* gene. The designed primer set *yst* Pr2a-b proved to be highly specific for the *yst* gene. In general, a PCR product with a consistent size of 163 bp was successfully amplified only from pathogenic strains either in multiplex PCR with 16S rDNA or in a separate amplification. Nonspecific products were never detected. In addition, neither nonpathogenic strains of *Y. enterocolitica* nor other bacterial species yielded any amplified product. Among the other *Yersinia* species tested, only two strains of *Y. kristensenii* gave rise to an amplicon of the same size.

Verification of the *yst* amplicon. In order to verify the authenticity of the amplified fragment, Southern hybridiza-

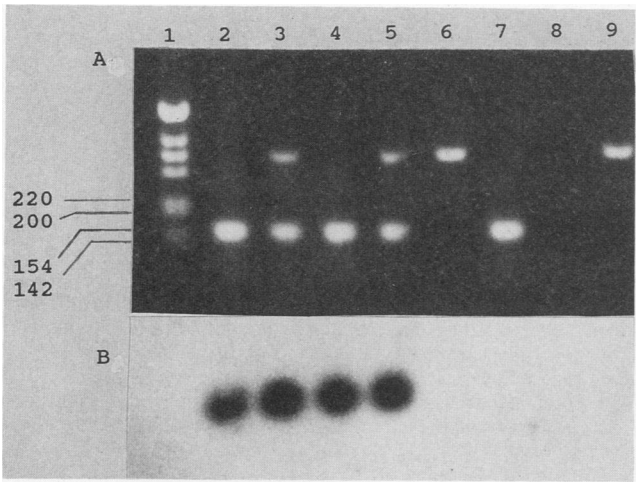

FIG. 2. (A) Gel electrophoresis pattern of *yst*-specific amplification by PCR (using primer set Pr2a-b). Lanes: 1, 1-kb ladder (Bethesda Research Laboratories); 2, *yst* product of *Y. enterocolitica* serovar O:8; 3, multiplex PCR of *Y. enterocolitica* serovar O:8; 4, *yst* product of *Y. enterocolitica* serovar O:3 (from seeded stool); 5, multiplex PCR of *Y. enterocolitica* serovar O:3 (from seeded stool); 6, multiplex PCR from normal stool (not containing *yst*-specific DNA); 7, *yst* of *Y. kristensenii*; 8, *yst* of nonpathogenic *Y. enterocolitica*; 9, multiplex PCR of nonpathogenic *Y. enterocolitica*. Numbers on the left are in base pairs. (B) Hybridization of Southern blot from the gel in panel A by using a [γ-^{32}P]ATP-labeled internal probe.

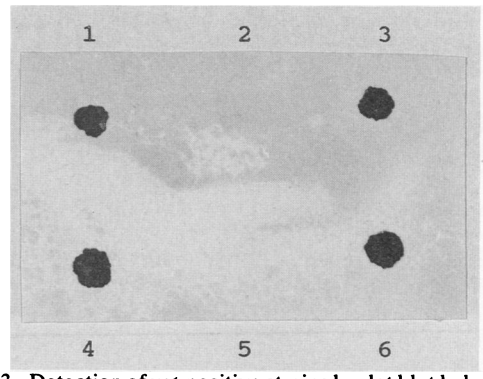

FIG. 3. Detection of *yst*-positive strains by dot blot hybridization on nylon membranes with the digoxigenin-dUTP-labeled internal *yst*-P probe. Dots: 1, 3, 4, and 6, *Y. enterocolitica* O:3; O:8; O:9; and O:5,27; respectively; 2, *Y. kristensenii*; 5, multiplex PCR of nonpathogenic *Y. enterocolitica* as a negative control.

tion was carried out with a 20-mer oligonucleotide probe. PCR products were transferred from the gel to nylon membranes and were hybridized with the [γ-^{32}P]ATP-labeled probe *yst*-P. This probe hybridized exclusively with the amplified *yst* fragment from all pathogenic strains of *Y. enterocolitica* but not with that of *Y. kristensenii*, confirming the specificity of the assay. In addition, we checked a nonradioactive detection system using digoxigenin-dUTP in a dot blot assay. PCR products were concentrated by selective alcohol precipitation (3), and an aliquot from each PCR was spotted onto a nylon membrane. Directly thereafter, hybridization was allowed to proceed for only 1 h after denaturation. Filters were briefly washed, and the hybrids were visualized as described above. Results were consistent with those obtained by Southern hybridization with the radioactively labeled probe (Fig. 3).

Fecal samples seeded with *Y. enterocolitica*. Thirty normal stool samples were examined for the presence of *yst*-positive *Y. enterocolitica*. While the 16S rDNA fragment was successfully amplified in all cases, the *yst* fragment was never visualized by gel electrophoresis, nor was it detected by the radiolabeled internal probe (Fig. 2B). Both fragments were generated, however, when the stool was seeded with a *Y. enterocolitica* serotype O:3 strain (Fig. 2A). The sensitivity of the assay was further evaluated by its ability to detect rather low numbers of the target cells. An amplicon corresponding to the *yst* gene could be visualized by agarose gel electrophoresis with as little as 10^2 CFU of *Y. enterocolitica* in the original fecal sample (data not shown).

Hospital strains and diarrheal stools. All five strains obtained from the Princess Alexandra Hospital and five of six strains from Royal Brisbane Hospital were positive for the *yst* gene in the test system that we used. Phenotypic virulence markers, including pyrazinamidase activity and salicin and esculin hydrolysis, were consistent with the results of the diagnostic assay (9). On the other hand, none of the diarrheal stool samples forwarded to our laboratory during this investigation contained the *yst* gene. This finding was consistent with the information that we later received from those laboratories that reported the absence of *Y. enterocolitica* in those samples, as detected by conventional techniques.

DISCUSSION

In a preliminary study to develop a highly specific PCR primer set for the *yst* gene, 10 pathogenic and 10 nonpathogenic strains of *Y. enterocolitica* were tested. Primer set Pr1a-b was designed to amplify almost the entire sequence of the 213-nucleotide-long *yst* gene. Although one major band appeared in all pathogenic strains tested, the appearance of nonspecific bands in both pathogenic and nonpathogenic strains indicated a lack of specificity of this primer set (9). So, another primer set, Pr2a-b, was designed to amplify a part of the *yst* gene. An amplified product that matched the size of the target sequence was consistently produced by the pathogenic strains of *Y. enterocolitica*, whereas no amplicons were generated from nonpathogenic strains. Therefore, a collection of 102 strains of *Yersinia* species of worldwide origin as well as many enterotoxigenic strains of other bacterial genera were screened. By using the diagnostic assay described here, the combination of the primer set Pr2a-b and the subsequent hybridization with the *yst* internal probe exclusively detected pathogenic strains of *Y. enterocolitica*. Moreover, certain serotypes, such as O:21 and O:3, have been reported to contain pathogenic and nonpathogenic strains (12, 22). The diagnostic assay described here was able to differentiate between typical (pathogenic) and atypical (nonpathogenic) strains of these serotypes, in that the *yst* gene was detected in the former but was absent from the latter. Although two strains of *Y. kristensenii* generated a fragment of the same size as the *yst* fragment, no hybridization signal was obtained with the internal probe. It is not unusual for there to be some mismatches within the internal sequence of the gene between the two organisms. An investigation is under way in our laboratory to sequence the gene from different strains of the two species and determine the degree of homology on both the nucleotide and the amino acid levels. None of the enterotoxigenic strains listed in

Table 2 produced any amplification products. Although there was a certain degree of homology between the *yst* gene and the heat-stable enterotoxin gene of *E. coli* at the 3′ end of the gene, with only six mismatches within the 21-oligonucleotide 3′ primer Pr2b, there were 14 mismatches within the 21 nucleotides of the 5′ primer Pr2a compared with the same target region of *E. coli* (see Fig. 3 of Delor et al. [6]). Thus, the possibility of amplifying a product from such a closely related organism was excluded.

The availability of DNA for amplification is a matter of concern, especially from fecal samples containing many inhibitory substances; also of concern is the subsequent sensitivity of the *Taq* polymerase. We amplified a stretch of the 16S rRNA gene using two universal primers along with the *yst* gene to test for false-negative results (Table 3). The 16S rDNA fragment was less efficiently amplified in the coamplification than it was in the absence of the *yst* target. This result may be attributed to the difference in the melting temperature of the primer pairs used (Table 3).

In addition, the amplification of a target sequence within a short period and the subsequent use of a nonradioactive assay would confirm the results of any PCR-mediated detection system. The use of a dot blot technique with a digoxigenin-dUTP-labeled probe produced the same results after 1 h of hybridization as those obtained by a Southern blot-radioactive probe detection system. Another major advantage of the nonradioactive detection system is that the same hybridization solution can be reused several times, which is more convenient for routine work in clinical laboratories.

In conclusion, the diagnostic assay described here is not only highly specific but is also capable of providing a clear-cut result on the same day that a specimen is submitted to the laboratory. The speed at which results are obtained compared with the speed at which results are obtained by conventional techniques will be a major advantage during an epidemic. In addition, the assay described here offers a powerful tool in epidemiological studies for establishing the precise etiology of an enteric infection.

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REFERENCES

1. **Aho, K.** 1983. *Yersinia* reactive arthritis. Br. J. Rheumatol. **22:**41–45.
2. **Bottone, E. J.** 1977. *Yersinia enterocolitica*: a panoramic view of a charismatic microorganism. Crit. Rev. Microbiol. **5:**211–241.
3. **Brow, M. A. D.** 1990. Sequencing with *Taq* DNA polymerase, p. 189–196. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and N. T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, London.
4. **Bufill, J. A.** 1989. *Yersinia enterocolitica* serotype O:3 sepsis after blood transfusion. N. Engl. J. Med. **320:**810.
5. **Cover, T. L., and R. C. Aber.** 1989. *Yersinia enterocolitica*. N. Engl. J. Med. **321:**16–24.
6. **Delor, I., A. Kaeckenbeeck, G. Wauters, and G. Cornelis.** 1990. Nucleotide sequence of *yst*, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and prevalence of the gene among pathogenic and nonpathogenic yersiniae. Infect. Immun. **58:**2983–2988.
7. **Denhardt, D. T.** 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23:**641.
8. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27:**682–685.
9. **Ibrahim, A., W. Liesack, and E. Stackebrandt.** 1992. Differentiation between pathogenic and nonpathogenic *Yersinia enterocolitica* strains by colony hybridization with a PCR-mediated digoxigenin-dUTP-labelled probe. Mol. Cell. Probes **6:**163–171.
10. **Jagow, J. A., and W. E. Hill.** 1988. Enumeration of virulent *Yersinia enterocolitica* by DNA colony hybridization using alkaline treatment and paper filters. Mol. Cell. Probes **2:**189–195.
11. **Kapperud, G., K. Dommarsnes, M. Skurnik, and E. Hornes.** 1990. A synthetic oligonucleotide probe and a cloned polynucleotide probe based on the *yopA* gene for detection and enumeration of virulent *Yersinia enterocolitica*. Appl. Environ. Microbiol. **56:**17–23.
12. **Kapperud, G., T. Nesbakken, S. Aleksic, and H. H. Mollaret.** 1990. Comparison of restriction endonuclease analysis and phenotypic typing methods for differentiation of *Yersinia enterocolitica* isolates. J. Clin. Microbiol. **28:**1125–1131.
13. **Krupp, G.** 1991. Direct sequence analysis of small rRNAs, p. 95–114. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Willey & Sons, Inc., Chichester, United Kingdom.
14. **Liesack, W., M. A. O. H. Menke, and E. Stackebrandt.** 1990. Rapid generation of vector-free digoxygenin-dUTP probes for nonradioactive hybridization using the polymerase chain reaction (PCR) method. Syst. Appl. Microbiol. **13:**255–256.
15. **Mason, P. J., and J. G. Williams.** 1985. Hybridization in the analysis of recombinant DNA, p. 113–137. *In* B. D. Hames and S. J. Higgins (ed.), Nucleic acid hybridization, a practical approach. IRL Press, Oxford.
16. **Miller, V. L., and S. Falkow.** 1988. Evidence of two genetic loci in *Yersinia enterocolitica* that can promote invasion of the epithelial cells. Infect. Immun. **56:**1242–1248.
17. **Nesbakken, T., G. Kapperud, K. Dommarsnes, M. Skurnik, and E. Hornes.** 1991. Comparative study of a DNA hybridization method and two isolation procedures for detection of *Yersinia enterocolitica* O:3 in naturally contaminated pork products. Appl. Environ. Microbiol. **57:**389–394.
18. **Noble, M. A., R. L. Barteluk, H. J. Freeman, R. Subramaniam, and J. B. Hudson.** 1987. Clinical significance of virulence-related assay of *Yersinia* species. J. Clin. Microbiol. **25:**802–807.
19. **Robins-Browne, R. M., M. D. Miliotis, S. Cianciosi, V. L. Miller, and S. Falkow.** 1989. Evaluation of DNA colony hybridization and other techniques for detection of virulence in *Yersinia* species. J. Clin. Microbiol. **27:**644–650.
20. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**437–491.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis (ed.).** 1989. Plasmid vectors, p. 1–110. *In* Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Schiemann, D. A.** 1984. Antigenic identity of *Yersinia enterocolitica* serotypes O:Tacoma and O:21. J. Clin. Microbiol. **20:**831–832.
23. **Schiemann, D. A.** 1989. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, p. 601–672. *In* M. P. Doyle (ed.), Food-borne bacterial pathogens. Marcel Dekker, Inc., New York.
24. **Schmitz, G. G., T. Walter, R. Seibl, and C. Kesseler.** 1991. Nonradioactive labelling of oligonucleotides *in vitro* with the hapten digoxigenin by tailing with terminal transferase. Anal. Biochem. **192:**222–231.
25. **Skurnik, M., and H. Wolf-Watz.** 1989. Analysis of the *yop*A gene encoding the Yop1 virulence determinants of *Yersinia* spp. Mol. Microbiol. **3:**517–529.
26. **Tipple, M. A., L. A. Bland, J. J. Murphy, M. J. Arduino, A. L.**

Panlilio, J. J. Farmer III, M. A. Tourault, C. R. Macpherson, J. E. Menitove, A. Grindon, P. S. Johnson, R. G. Strauss, J. A. Bufill, P. S. Ritch, J. R. Archer, O. C. Tablan, and W. R. Jarvis. 1990. Sepsis associated with transfusion of red cells contaminated with *Yersinia enterocolitica*. Transfusion **30:**207–213.

27. **Woese, C. R., R. Gutell, R. Gupta, and H. F. Noller.** 1983. Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiol. Rev. **47:**621–669.
28. **Wormser, G. P., and G. T. Keusch.** 1981. *Yersinia enterocolitica*: clinical observations, p. 84–93. *In* E. J. Bottone (ed.), *Yersinia enterocolitica*. CRC Press, Inc., Boca Raton, Fla.
29. **Zink, D. L., J. C. Feeley, J. G. Wells, C. Vandrzant, J. C. Vickery, W. D. Roof, and G. A. O'Donovan.** 1980. Plasmid-mediated tissue invasiveness in *Yersinia enterocolitica*. Nature (London) **283:**224–226.