We have developed a fluorescence resonance energy transfer-based (FRET) assay to detect ciprofloxacin resistant (Cpr) mutants of the biothreat agent Yersinia pestis. We selected spontaneous mutants of the attenuated Y. pestis KIM 5 strain that were resistant to at least 1 mg/ml Cp. DNA sequencing of gyrA encoded by sixty-five of these mutants revealed that all isolates contained one of four different point mutations within the quinolone resistance-determining region of gyrA. We developed a FRET assay that detected all of these mutations using a single pair of fluorescent probes with sequences complementary to the wild type Y. pestis gyrA sequence. Melting peak analysis revealed that the probe-PCR product hybrid was less stable when amplification occurred from any of the four mutant templates. This instability resulted in the PCR product obtained from the Cpr Y. pestis strains displaying a 4-11OC shift in probe melting temperature. Following optimization of the reaction conditions we were able to detect approximately 10 pg of purified wild type template DNA or the presence of approximately 4 CFU of Y. pestis KIM 5 wild type or Cpr mutants in crude lysates. Taken together, our results demonstrate the utility of FRET-based assays to detect Cpr mutants of Y. pestis that is both sensitive and rapid.
Detection of Ciprofloxacin-Resistant *Yersinia pestis* by Fluorogenic PCR Using the LightCycler

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We have developed a fluorescence resonance energy transfer (FRET)-based assay to detect ciprofloxacin resistant (Cp') mutants of the bioterrorism agent *Yersinia pestis*. We selected spontaneous mutants of the attenuated *Y. pestis* KIM 5 strain that were resistant to a ciprofloxacin (CIP) concentration of at least 1 µg/mL. DNA sequencing of *gyrA* encoded by 65 of these mutants revealed that all isolates contained one of four different point mutations within the quinolone resistance-determining region of *gyrA*. We developed a FRET-based assay that detected all of these mutations by using a single pair of fluorescent probes with sequences complementary to the wild-type *Y. pestis* *gyrA* sequence. Melting peak analysis revealed that the probe-PCR product hybrid was less stable when amplification occurred from any of the four mutant templates. This instability resulted in the PCR product obtained from the Cp* Y. pestis* strains displaying a 4 to 11°C shift in probe melting temperature. Following optimization of the reaction conditions, we were able to detect approximately 10 pg of purified DNA from wild-type *Y. pestis* KIM 5 or Cp' mutants in crude lysates. Taken together, our results demonstrate the utility of FRET-based assays for detection of Cp' mutants of *Y. pestis*. This method is both sensitive and rapid.

Resistance to antibiotics has become a major concern for the medical community over the past several years (13, 14, 16). Many organisms have become resistant to the common "drug of choice" used to treat the disease. A few examples are methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci (18), and multiple-drug-resistant *Mycobacterium tuberculosis* (19), as well as organisms causing many enteric diseases. One of the current antibiotics that is effective in treating bacterial infectious diseases is ciprofloxacin (CIP), a fluorinated quinolone that blocks DNA replication through inhibition of gyrase activity (2, 24). Resistance to CIP does occur and is usually mediated by point mutations in DNA gyrase or, less commonly, through membrane alterations that reduce drug entry into the bacteria (28).

A critical piece of information necessary for the treatment of any bacterial disease is the antibiotic sensitivity profile of the infectious agent. Classically the sensitivity profile has been determined by growth of the organism in the presence of the antibiotic either in agar diffusion assays or by incubation of the organism in various concentrations of the drug for determination of the MIC. Both of these methods depend on growth of the organism after its initial isolation and are therefore time-consuming. DNA probe-based detection of antibiotic resistance offers the potential for increased speed. Among DNA-based techniques, PCR offers the best opportunity for speed, sensitivity, and specificity.

Recently it has become possible to couple PCR with real-time detection of the amplification product by use of fluorescent probes, thus eliminating the necessity to analyze the reaction product by gel electrophoresis. Fluorescence resonance energy transfer (FRET) is one of the available chemistries that can be used to detect the PCR product in these reactions. Roche Diagnostics has adopted this chemistry for its "Hybridization Probes" technology (5). Two DNA probes are used to bind to the amplification product when FRET chemistry is used to specifically detect the amplification product. The two light-activated molecules are positioned in close proximity at the 3' and 5' termini of the probes such that fluorescence increases as more PCR product accumulates and the two labeled probes bind next to each other.

The increase in antibiotic resistance coupled with the threat of modification of agents of biological warfare have prompted us to develop a hybridization probe assay for the detection of CIP resistance (Cp') in *Yersinia pestis*. We chose *Y. pestis* as a model for our initial development of a Cp' assay for three reasons. First, it is the etiologic agent of a disease that has high potential for use as a biological terrorism or biological warfare agent (27). Second, it is likely that any biological used as a weapon would be made antibiotic resistant. Third, *Y. pestis* is relatively slow growing, and therefore the increased speed of DNA-based antibiotic resistance detection would improve our ability to properly treat infected individuals. Although our development of the assay used *Y. pestis* as a model, the method should be broadly applicable to the detection of antibiotic resistance encoded by point mutations in other organisms.

**MATERIALS AND METHODS**

**Cultivation of bacteria, isolation of Cp' mutants, and antibiotic sensitivity testing.** The bacterium used in this study was an avirulent, pigmentation-negative (pem-negative) mutant of *Y. pestis* KIM 5 and was obtained from Susan Strelay, Department of Microbiology and Immunology, University of Kentucky, Lexington, Ky. Bacteria were grown on brain heart infusion agar (BHI; Difco Laboratories, Detroit, Mich.) plates for 48 h at 30°C or overnight in BHI broth at 30°C with aeration. After cultivation, the bacteria were collected, washed twice with phosphate-buffered saline (150 mM NaCl, 1.7 mM KH₂PO₄, 5 mM Na₂HPO₄ [pH 7.4]) and suspended to yield a concentration of 10¹⁴ CFU/ml. For selection of
Cp' mutants; a 100-μl sample of washed bacteria was plated on BHI agar plates containing, to 10 μg of CIP (Bayer, Leverkusen, Germany/ml. Cp' mutants appeared after 48 to 72 h of incubation at 30°C. Colonies were purified by streaking on fresh BHI agar plates containing the appropriate concentration of CIP. The MIC for CIP was determined by using E Test (AB Biodisk, Piscataway, N.J.) antibiotic strips containing a continuous gradient of CIP. The E Test was performed by spreading 100 μl of bacterial culture onto BHI agar plates without antibiotics. After 16 h of incubation at 30°C, the MIC was read according to the manufacturer's instructions.

DNA isolation, gene amplification, and sequencing. Total-cell DNA was extracted from 5-mI cultures of Y. pestis or Cp' mutants with the Puregene DNA Isolation Kit (Genta Systems, Minneapolis, Minn.). Initially, primers to amplify the quinolone resistance-determining region (QRDR) (30) of gyrA were selected from the published Echerichia coli sequence (GenBank accession number AE00312) (3) using Primerselect software (Lasergene, Madison, Wis.). PCR amplification primers gyrA51 and gyrA31 were ATGAGGCACCTGGGAGAG and GTCTTTCATGCTTCTTTACAGTG, respectively. We used PCR and direct DNA sequencing to identify Y. pestis gyrA mutations. PCR was carried out with AmpliTaq Gold (Perkin-Elmer, Foster City, Calif.) according to the manufacturer's directions. Initial activation of AmpliTaq Gold and denaturation of template DNA were carried out at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 7 min. Amplification products were examined for size and purity by fractionation on a 1% agarose gel. PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen, Valencia, Calif.). Nucleotide sequence determination was performed by the dideoxy chain-termination method using the Applied Biosystems International (ABI, Foster City, Calif.) PRISM dideoxyribonucleic acid Terminator Sequencing Kit with AmpliTaq Gold DNA polymerase. DNA sequencing reactions were analyzed on an ABI 377XL automated DNA sequencer. Sequence data were edited and assembled into contiguous sequences using the Sequencer program (Gene Codes, Ann Arbor, Mich.). The Y. pestis KIM 5 wild-type gyrA nucleotide sequence was determined using the gyrA51 and gyrA31 primers used in the initial PCR. The DNA sequences of the mutant gyrA genes were determined using oligonucleotide primers gyrA51seq and gyrA31seq, which had the sequences AAATAACACCGGTTTTATTGG and TGAGTTCCATCAGCCCTTCAATG, respectively. We used PCR and direct DNA sequencing with the Sequencer program (Gene Codes, Ann Arbor, Mich.). The Y. pestis KIM 5 gyrA gene sequence was determined using the gyrA51seq and gyrA31seq primers used in the initial PCR. The DNA sequences of the mutant gyrA genes were determined using oligonucleotide primers gyrA51seq and gyrA31seq, which had the sequences AAATAACACCGGTTTTATTGG and TGAGTTCCATCAGCCCTTCAATG, respectively. We used PCR and direct DNA sequencing with the Sequencer program (Gene Codes, Ann Arbor, Mich.). The Y. pestis KIM 5 gyrA gene sequence was determined using the gyrA51seq and gyrA31seq primers used in the initial PCR. The DNA sequences of the mutant gyrA genes were determined using oligonucleotide primers gyrA51seq and gyrA31seq, which had the sequences AAATAACACCGGTTTTATTGG and TGAGTTCCATCAGCCCTTCAATG, respectively. We used PCR and direct DNA sequencing.

RESULTS

Isolation of Cp' Y. pestis mutants. We performed 11 independent experiments to isolate Cp' mutants. The average mutation frequency observed for all of these experiments was 2.6 × 10⁻¹⁰. These mutant isolations resulted in 682 Cp' Y. pestis KIM 5 strains. We chose 65 mutants at random for further single-colony isolation and characterization. We performed initial experiments to determine the influence of plating cell density on the CIP MIC observed by using E Test antibiotic sensitivity strips. Inoculation of BHI agar plates with approximately 10⁷ or 10⁸ CFU per plate produced significantly higher or lower observed MICs, respectively. Accordingly, we chose to plate Y. pestis KIM 5 at 10⁷ CFU per plate in order to determine the observed MIC for CIP in all later experiments. Our observed CIP MIC for the wild-type Y. pestis strain KIM 5 was 0.026 μg/ml. For all the Cp' mutants, MICs at least 40 times greater than that for the wild type, i.e., between 1.1 and 4.6 μg/ml, were observed (Fig. 1B).

Sequencing of wild-type Y. pestis and Cp' mutants. We obtained a single, approximately 630-bp fragment of DNA from the Y. pestis KIM 5 genome using oligonucleotide primers gyrA51 and gyrA31 in PCRs. After determination of the sequence of this fragment on both strands of DNA, a BLASTN (1) search of GenBank (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) revealed that our sequence had a high degree of homology (probability value, 1e⁻¹³⁹) with Serratia marcescens gyrA (GenBank accession number AF052260) (26). The final 492-bp Y. pestis KIM 5 gyrA sequence was 88% identical to S. marcescens gyrA at the nucleotide level, suggesting that it was the plague homologue of the gyrA gene. Further evidence was obtained by performing protein searches of the nonredundant GenBank database. The protein database search revealed that the putative protein product translated from our DNA sequence was 96% identical over a 164-amino-acid overlap with S. marcescens GyrA (AAC68576) (26) as well as many other E. coli GyrA homologues. Our analysis of the predicted Y. pestis KIM 5 GyrA sequence obtained from the PCR product using Megalign (Lasergene) revealed that the nucleotide sequence changes encoded six conservative amino acid substitutions compared to S. marcescens GyrA. Our results revealed that the Y. pestis KIM 5 GyrA sequence within the QRDR was...
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DNA-BASED Cp' ASSAY 3651

A.

M1  ACCACCCCGCATGATGACGGCGGGCTT  Gly to Asp at pos 81
M2  ACCACCCCGCATGATGACGGCGGGCTT  Gly to Cys at pos 81
M3  ACCACCCCGCATGATGACGGCGGGCTT  Ser to Ile at pos 83
M4  ACCACCCCGCATGATGACGGCGGGCTT  Ser to Arg at pos 83
WT  ACCACCCCGCATGATGACGGCGGGCTT  Wild Type

FIG. 1. Nucleotide sequence and protein changes in Y. pestis Cp' mutants. (A) DNA sequences of wild-type (WT) Y. pestis gyrA and the four point mutants, identified as M1 through M4, corresponding to their designations in the text. Underlined nucleotides in the WT sequence denote the FRET assay probe 1. Nucleotide substitutions in their designations in the text. Underlined nucleotides in the WT sequence and are labeled LC3 and LC4. Probe 1 and probe 2 are shown between LC3 and LC4. The starbursts at the 3' and 5' termini of each probe indicate the melting temperatures of each probe.

B.

E. coli gyra

Table 1. MIC for mutants 3651

<table>
<thead>
<tr>
<th>Mutant</th>
<th>MIC (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>M1</td>
<td>4.10 (1.37)</td>
</tr>
<tr>
<td>M2</td>
<td>4.35 (1.09)</td>
</tr>
<tr>
<td>M3</td>
<td>4.31 (0.39)</td>
</tr>
<tr>
<td>M4</td>
<td>4.35 (1.09)</td>
</tr>
</tbody>
</table>

Since other gene products such as GyrB (8, 12, 31) and ParC (6, 11, 20) have been shown to be involved in resistance to CIP, we amplified the QRDRs of these genes from a randomly selected group of our Cp' mutants and compared the DNA sequences for both Y. pestis KIM 5 gyrB and parC. The DNA sequences of the wild type Y. pestis KIM 5 gyrB and parC were determined as described in Materials and Methods. To confirm that the genes we amplified did encode the expected proteins, our nucleotide sequences were translated into predicted proteins and used to search the GenBank protein database. The product protein predicted by translation of the wild-type Y. pestis gyrB sequence was highly homologous (probability value, 1e^-102) with E. coli GyrB (AAC76722) (3). The entire protein sequence predicted by translation of Y. pestis KIM 5 parC was 86% identical with the E. coli ParC sequence (P20882) (3), amino acids 12 through 394. Accordingly, the putative function of the proteins encoded by our PCR product obtained after Y. pestis gyrB and parC amplification was confirmed by protein homologies in the database. The DNA sequence of gyrB carried by 12 of our 65 Y. pestis Cp' mutants was determined from randomly selected strains regardless of observed MIC and was found to be identical with the wild-type sequence. Similarly, the parC sequence encoding the QRDR was determined for 36 of the 65 Cp' mutants and was found to be identical to the wild-type DNA sequence.

Amplification and detection of gyrA in the LightCycler. We designed PCR primers and hybridization probes for detection of Y. pestis KIM 5 gyrA as shown in Fig. 2. Oligonucleotide probes are represented by arrows above or below the Y. pestis gyrA sequence and are labeled LC3 and LC4. Probe 1 and probe 2 are shown between LC3 and LC4. The starbursts at the 3' and 5' termini of each probe indicate the melting temperatures of each probe. The positions of the four point mutations described in Fig. 1A are indicated (boldfaced, underlined letters) in the wild-type DNA sequence below probe 1. The Tm of probe 1 was chosen to be less than that of probe 2 such that detection of melting of the probes from the PCR product would be dependent on the stability of probe 1 with the product.
primers LC3 and LC4 were designed to amplify a 261-bp region of gyrA that included all of the point mutations within the QRDR we had detected in our Cp' mutant isolation (Fig. 1). As shown in Fig. 2, the FRET detection probe 1 was chosen to have the same sequence as wild-type *Y. pestis* gyrA and to encompass all four of the point mutations within the gene that resulted in Cp'. FRET probe 2 was selected such that the 5' end of the oligonucleotide containing the LightCycler-Red-640 label was positioned 1 bp away from the probe 1 3' end (Fig. 2). The predicted melting temperatures (*T*m) for probe 1 and probe 2 were 59.6 and 64.9°C, respectively. We reasoned that we would be able to detect the mutant genes through melting curve analysis by using a single probe, since probe 1 was based on the wild-type allele and included the region where all of the Cp' point mutations occurred in gyrA.

We tested the ability of the hybridization probe assay to detect *Y. pestis* gyrA sequences using purified genomic DNA as a template. Melting peak analysis revealed that the change in fluorescence with the change in time (*dF/dT*) versus hybrid temperature at the various DNA concentrations. The 0.001-ng template reaction mixture was indistinguishable from the no-template control (water). NTC, no-template control.

hybrid with probe 1 than did the wild-type gyrA sequence. The largest difference in melting peak was seen with the cytosine-to-adenosine mutation (M4 in Fig. 1A), which was closest to the 3' fluorescent label on probe 1. Interestingly, the most-stable probe 1-mutant PCR product hybrid was formed with the guanine-to-thymine transversion mutant (Fig. 1A and 4). This mutation was located 1 bp 5' to the mutation in the strain that formed the least-stable hybrid with probe 1 (M3 in Fig. 1A). However, the two mutations that were the most distal to the 3' end of probe 1 (M1 and M2 in Fig. 1A) had an intermediate *T*m (Fig. 1A). In all experiments the *Ar* (defined as the *T*m of the wild type - the *T*m of the mutant) was also consistent and varied less than 0.5°C.

**Colonhy assay for Cp' Y. pestis.** In order to test the utility of our FRET assay for identification of Cp' *Y. pestis*, we performed the assay on crude lysates of bacteria. Initially, we used boiled lysates from a single colony suspended in 100 μl of water as the template in these reactions without any further growth of the cells. We found that we could identify the Cp'
mutant M4. Bacteria used in the reactions or Cp
Y. pestis
of wild-type
control sample. The curve labeled "water" represents the no-template
concentrations of organisms that might be obtained after the 3-h
growth period. The curve labeled "undil." contained 4 X 10^9 CFU. Suspensions
are labeled. The sample labeled "undil." represents the reaction ob-
harvested. Shown is a melting peak analysis of various concentrations
Single colonies were grown in broth for 3 h at 30°C before being
allowed us to detect the presence of Y. pestis gyrA
produced, we analyzed a 10-µl sample of the Y. pestis KIM 5
reaction products on agarose gels. We did not detect any gyrA
PCR product in the reaction products produced with the 10^-5
and 10^-6 dilutions (data not shown).

FIG. 5. Detection of Cp' mutations in crude whole-cell lysates. Single colonies were grown in broth for 3 h at 30°C before being harvested. Shown is a melting peak analysis of various concentrations of wild-type Y. pestis or Cp' mutant M4. Bacteria used in the reactions are given above each melting peak. Curves obtained with each dilution
are labeled. The sample labeled "undil." represents the reaction ob-
tained with undiluted bacterial suspensions. The 2-µl sample used in the
FRET PCR labeled "undil." contained 4 X 10^9 CFU. Suspensions of bacteria were diluted before lysis by boiling to simulate different concentrations of organisms that might be obtained after the 3-h
growth period. The curve labeled "water" represents the no-template
control sample.

DNA-BASED Cp' ASSAY

Although Cp' in Y. pestis is currently of no concern to public health officials, the organism is of major concern as a potential agent for biological warfare or bioterrorism (27). Given that
CIP is a new-generation antibiotic under consideration for inclusion in a national antibiotic stockpile (9), a rapid method for the detection of Cp' agents of bioterrorism is highly desirable. We have developed a simple and rapid PCR assay to detect Cp' using FRET probes and the Roche Diagnostics LightCycler. The assay was able to detect four different point mutations within Y. pestis KIM 5 gyrA using a single probe pair with as few as approximately 2,100 copies of purified target sequence. Cp' Y. pestis could also be detected in crude lysates prepared from fresh colonies isolated on petri plates at levels below 10 CFU per reaction. The difference in detection level between pure and crude templates is most likely due to the tendency of Y. pestis to grow as chains under culture conditions similar to those used here (L. E. Lindler, unpublished data). Using our assay, we were able to determine if the bacteria growing on solid medium were Cp' within 4 h using melting peak analysis. Furthermore, the FRET assay was able to reliably identify Cp' Y. pestis at CFU concentrations of 4 to 40,000 per reaction. This finding is particularly important if the method is to be useful in a clinical laboratory, since the assay will identify Cp' bacteria over a wide range of template concentrations, thus reducing the possibility of false-negative re-
actions.

Given that FRET depends on the interaction of two fluoro-
chromes in proximity with each other, it might be expected that the location of the base mismatch within probe 1 would strongly influence the melting peak. However, our results indicate that the position of the mismatch within probe 1 does not greatly influence this stability. This fact was suggested by our observation that mutant M4 and M3 produced the largest and smallest T_m values in our FRET assay and were located only 1 bp apart. The stability of these mutant templates was especially noteworthy, since both M4 and M3 are located nearest the 3' terminus of probe 1 and therefore might be expected to decrease the excitation of probe 2. If the position of the mismatch within the probe 1 sequence were a major factor in probe-template stability, then mutants M1 and M2 (Fig. 1A) might be expected to reduce the observed T_m to a lesser degree
than mutant M3. However, the melting peaks of M1, M2, and
M3 were very similar, further suggesting that the position of the mismatch did not influence probe 1 binding to the PCR product to any great degree. Taken together, our results sug-
gest that the greatest influence on the observed melting peak
using the FRET assay is due to the sequence of the base pair
mismatch rather than the position of the mismatch within the

Currently, the standard method of determining Cp' by
agar diffusion tests. This method requires the isolation of the pathogen followed by an extra day of incubation with disks or strips. DNA-based methods for Cp' detection, such as mismatch amplification mutation assay (MAMA) combined with
DNA sequencing (32) and single-stranded confirmation poly-
morphism (23), have been developed. All of these methods require electrophoresis of reaction products to determine if a mutant allele of a cellular gyrase is encoded by the isolate. Also, both of the techniques above require equipment that does not easily lend itself to use in a clinical laboratory environment. Most recently, Wilson et al. (29) developed a 5' nuclease assay to detect Cpf in Campylobacter jejuni by PCR allelic discrimination (AD). These researchers were able to distinguish mutations at codon 86 by use of a pair of fluorescent probes and comparison of binding of those probes with either mutant or wild-type PCR products. Although this assay was shown to be sensitive to the femtogram level of template DNA, it has not been tested for the ability to distinguish Cpf mutant strains from sensitive strains using crude whole-cell lysates as we have demonstrated here. Furthermore, we believe the FRET-based assay is more applicable because the use of a single pair of FRET probes allows the detection of four different linked point mutations in gyrA. In contrast, detection of the point mutations described here would require at least five different probes to be developed for an AD assay, assuming that one wild-type probe could be paired with a probe that binds to each individual point mutation in gyrA.

The mutations in Y. pestis gyrA all occurred at position 81 or 83 relative to the E. coli protein sequence. Although we did not determine the sequences of gyrB and parC for every Cpf mutant we identified, our random sampling of these gene sequences suggests that they were most probably wild type in the mutants we characterized. Specifically, the observed MICs for all of the individual members of our four mutant classes were similar, and secondary alterations in gyrB and/or parC have been shown to result in increased levels of Cpf (7, 11, 20). Taken together, our random DNA sequencing of known secondary mutation sites and our MIC data strongly suggest that the Y. pestis Cpf strains we characterized did not encode mutations in the gyrB and/or parC ORDR.

Six different amino acid substitutions in E. coli GyrA have been identified in Cpf strains following in vitro selection (4, 10, 22, 30). Among our 65 Y. pestis Cpf mutants we found only two amino acid positions in GyrA that had been changed. The substitutions in Y. pestis GyrA were Gly-81 to Asp or Cys and Ser-83 to Ile or Arg. All of the amino acid substitutions we identified in Y. pestis GyrA have been found in other organisms (4, 15, 20, 23, 25, 30). Based on the results of our characterization of 65 Y. pestis Cpf mutants, the most common site of mutation in GyrA is Gly-81. Other studies have indicated that Ser-83 is the most common hotspot for changes in GyrA. Alternatively, this finding may be due to the limited number of Cpf isolates we characterized.

In summary, we have developed a pair of FRET probes that can easily detect four closely linked point mutations in Y. pestis gyrA by use of melting peak analysis. This FRET-based Cpf detection method is sensitive, reproducible, and applicable over a wide range of template concentrations. In order for this assay to be useful in a clinical laboratory setting, it should be possible to perform the analysis as quickly after initial isolation of the organism as possible. We have demonstrated that this is possible by use of crude whole-cell lysates as templates in our reactions. Further testing of specificity is planned for future experiments. However, currently we envision that the FRET assay described here could be incorporated into a general DNA-based identification panel that would include Y. pestis-specific primers. Accordingly, it may be possible in the future to identify organisms and determine their antibiotic sensitivity profiles simultaneously. The development of our FRET-based Cpf assay is a first step toward this goal.

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REFERENCES


