Optimal Probe Length and Target Location for Electrochemical Detection of Selected Uropathogens at Ambient Temperature[▽]

Mitra Mastali,¹ Jane T. Babbitt,¹ Yang Li,² Elliot M. Landaw,³ Vincent Gau,⁴
Bernard M. Churchill,² and David A. Haake^{1,5*}

Departments of Medicine,¹ Urology,² and Biomathematics,³ David Geffen School of Medicine at UCLA, Los Angeles, California 90095; GeneFluidics, Monterey Park, California 91754⁴; and Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California 90073⁵

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We have previously demonstrated the clinical validity of the rapid detection of uropathogens by use of a DNA biosensor. This assay involves the hybridization of capture and detector probe pairs with bacterial 16S rRNA target molecules to form a DNA-RNA sandwich on the sensor surface. Horseradish peroxidase-conjugated antibody binds to the detector probe to enzymatically amplify the hybridization signal. These previous studies involved the hybridization of bacterial 16S rRNA target sequences with 35-mer oligonucleotide probe pairs at 65°C. Achievement of point-of-care technology will be greatly facilitated by ambient-temperature detection. The purpose of this study was to examine the effects of probe length and target location on signal intensity using hybridization temperatures of 20 to 25°C. Signal intensity was found to vary dramatically with hybridization location in the species-specific bulge region of 16S rRNA helix 18. Probe pairs of as short as 10 nucleotides in length were able to produce a significant electrochemical signal, and signal intensity was correlated with probe length for probes of 10 to 20 nucleotides in length. The sensitivity of the *Escherichia coli*-specific 15-mer probe pairs was approximately 330 cells. These shorter probes allowed differentiation of *Klebsiella pneumoniae* from *Proteus mirabilis* 16S rRNA target sequences differing by a single nucleotide. A panel of oligonucleotide probe pairs ranging from 11 to 23 nucleotides in length was able to distinguish among seven groups of urinary tract pathogens. In conclusion, we have developed short oligonucleotide probe pairs for the species-specific identification of uropathogens at ambient temperature by use of an electrochemical sensor.

There is an urgent need to develop rapid methods for microbial detection and identification for patient specimens. Currently, it is typical for 48 to 72 h to elapse between specimen collection and the delivery of the microbiology report. Awareness of this delay encourages physicians to select antibiotic therapy “empirically” based on the nature of the infection and historical antibiotic susceptibility data but without direct information about the actual pathogen causing the patient’s infection. Development of technology for rapid, accurate, and cost-effective bacterial detection systems would revolutionize the diagnosis, prevention, and treatment of infectious diseases. For certain clinical scenarios, such as the diagnosis of urinary tract infection, biosensors that directly detect nucleic acid targets would have distinct advantages.

Bacterial pathogen detection using electrochemical biosensors has been demonstrated using a variety of approaches (3). In each approach, a biological recognition process is linked to an electrode transducer. For example, glucose sensors use electrodes impregnated with enzymes, such as glucose oxidase, to couple the oxidation of glucose to a measurable current flow (19). The simplicity and low power requirements of such devices have allowed the fabrication and mass marketing of blood glucose sensors as compact, inexpensive, handheld devices (12). Because of their speed, sensitivity, accuracy, and versatility, electrochemical sensors are now widely used in emer-

gency departments to measure blood gases, electrolyte concentrations, and a variety of other substrate characteristics (5). Recently, a variety of approaches have successfully been demonstrated for the electrochemical detection of nucleic acid target molecules (4, 20). These approaches link DNA-DNA or DNA-RNA hybridization events to current output by use of oligonucleotide probes immobilized on an electrode surface. Nucleic acid detection assays involving the amplification of hybridization signals through enzyme tracer molecules have the potential for ultrasensitive detection (13). Enzymes such as alkaline phosphatase or horseradish peroxidase convert electrochemically inactive substrate to an electroactive product that can be detected amperometrically. In this way, each captured enzyme molecule (i.e., binding event) generates thousands of detectable products. The current signal is proportional to the amount of the bound enzyme (and hence to the analyte concentration in the sample).

The sensitivity of the electrochemical biosensor approach is enhanced when the target is present in high copy numbers per bacterial cell. Bacteria contain 10⁴ to 10⁵ ribosomes per cell, containing the 23S, 16S, and 5S rRNA molecules. The molecular basis for our sensor technology is the recognition of species-specific “signature” sequences located on oligonucleotide probe-accessible regions of the 16S rRNA molecule (6). The detection strategy is an electrochemical sandwich assay in which target 16S rRNA is bound by both a capture and a detector probe (7, 8). The capture probe anchors the target to the sensor and the detector probe provides a means for recognizing target bound on the sensor surface. The detector probe links the surface via the target and capture probe to

* Corresponding author. Mailing address: Division of Infectious Diseases, 111F, VA Greater LA Healthcare, Los Angeles, CA 90073. Phone: (310) 268-3814. Fax: (310) 268-4928. E-mail: dhaake@ucla.edu.

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horseradish peroxidase for electrochemical signal amplification. We have demonstrated the analytic validity of this sensor strategy for the electrochemical detection of uropathogens in clinical urine specimens (9). Probe refinements, including elimination of the gap between the capture and detector probe hybridization sites and the location of the fluorescein modification of the detector probe, led to further improvements in signal intensity and sensitivity (10). Our previous studies involved 35-mer capture and detector probes and hybridization with the 16S rRNA target at 65°C (7–10, 17). In this study, experiments were performed at room temperature, which was typically in the range of 20 to 25°C. Despite the variation in temperature within this range, we obtained reproducible detection of 16S rRNA target molecules by use of capture and detector probes as short as 10 nucleotides in length with excellent discrimination between uropathogen species.

MATERIALS AND METHODS

Bacterial strains and cultivation. The following American Type Culture Collection (ATCC) strains were obtained from the UCLA Clinical Microbiology Laboratory: *Escherichia coli* strain 35218, *Klebsiella pneumoniae* strain 13883, *Klebsiella oxytoca* strain 49131, *Enterobacter aerogenes* strain 13048, *Enterobacter cloacae* strain 13047, *Proteus mirabilis* strain 12453, *Pseudomonas aeruginosa* strain 10145, *Citrobacter freundii* strain 8090, and *Enterococcus faecalis* strain 49532. The following additional strains of uropathogenic bacteria were obtained from the UCLA Uropathogen Specimen Bank: *E. coli* strain Ec103, *K. pneumoniae* strain Kp101, and *P. mirabilis* strain Pm193. Isolation of uropathogens from clinical urine specimens was approved by the UCLA and VA Greater Los Angeles Healthcare System Institutional Review Boards. The identities of all clinical strains were determined by standard biochemical assays and verified by 16S rRNA gene sequencing. The 16S rRNA genes were PCR amplified with universal primers 8UA and 1485B (2). The amplified product was purified by using the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA) and directly sequenced using primer pairs 8UA/907B and 774A/1485B as described previously (16). DNA sequencing was performed at the W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, CT). Isolates were inoculated into Brucella broth with 15% glycerol (BBL, Maryland) and were stored at –70°C. All experiments reported here involved bacteria grown overnight in Bacto tryptic soy broth (Becton, Dickinson, Sparks, MD), inoculated into tryptic soy broth, and grown to logarithmic phase as measured by optical density at 600 nm. The concentration of the logarithmic-phase specimens was determined by serial plating, typically yielding 10^7 to 10^8 bacteria/ml.

Oligonucleotide probes. Oligonucleotide probes were synthesized by MWG Biotech (High Point, NC) and Sigma (St. Louis, MO). Capture probes were synthesized with a 5' biotin modification. Detector probes were synthesized with 3' fluorescein modifications. Oligonucleotide probe pairs were designed to hybridize with species-specific regions of the 16S rRNA molecules of *E. coli*, *E. faecalis*, *P. mirabilis*, *K. pneumoniae*, and *P. aeruginosa*. Oligonucleotides were also designed as capture and detector probes for the family *Enterobacteriaceae* and as universal bacterial probes. The sequences of all oligonucleotide probes used in this study are shown in Table 1.

Sensor characterization and surface functional-layer preparation. Microfabricated electrochemical sensor arrays with an alkanethiolate self-assembled monolayer were obtained from GeneFluidics (Monterey Park, CA). Self-assembled monolayer integrity was confirmed by cyclic voltammetry (1) using a 16-channel potentiostat (GeneFluidics). After cyclic voltammetric characterization, sensor surfaces were functionalized with streptavidin and with biotinylated capture probes as previously described (10).

Amperometric detection of bacterial 16S rRNA. The detailed procedures for cell preparation and lysis, hybridization of target rRNA with detector and capture probes, and amperometric detection of species-specific rRNA have been described elsewhere (10), with the exception that in the present experiments hybridizations were carried out at room temperature (20 to 25°C).

Experiments were performed on ATCC strains to verify probe specificity using a 16-sensor array in which the UNI795C, EB1275C, EC454C, KP451C, PM2107C, PA132C, and EF220C 5'-biotinylated capture probes (defined in Table 1) were tested in duplicate. The two remaining sensors in the array served as negative controls (using capture probe UNI795C in 1 M phosphate buffer, pH

7.4). Bacterial lysates were combined with a mixture of the following 3'-fluorescein-labeled detector probes: UNI776D, EB1252D, EC439D, KP440D, PM189D, PA132D, and EF200D. Detector probe mixture without bacterial lysate was added to the negative-control sensors.

Statistical analysis. The variance in signal intensity measurements obtained using the electrochemical sensor was evaluated by comparing results from duplicate experiments. Analysis of variance across all experiments by use of logarithms (base 10) of the signals indicated that variability in the log domain was somewhat increased for small signal intensities, as reported in a previous study (10). An overall pooled estimate of the standard deviation (SD) was 0.099 log units for signals of less than 100 nA and 0.062 for signals of greater than 100 nA. As a conservative measure, we used the SD for low signals in all statistical analyses, so that 0.099 (with 93 degrees of freedom) became the standard error of the difference between duplicate means in each *t* test comparing means in the log domain.

RESULTS

The effect of probe hybridization location on signal intensity was examined. Experiments were performed at room temperature and involved a series of capture and detector probes varying from 8 to 14 nucleotides in length to achieve a melting temperature of between 39 and 46°C as determined by the base-stacking method (14, 15). Figure 1A shows the current output (in nanoamperes) measured using a series of melting-temperature-comparable capture and detector probes hybridized to 16S rRNA released from 9.1×10^6 *E. coli* cells. Numbers on the horizontal axis refer to the position of the first nucleotide of the capture probe hybridization location. Probe pairs with junctions hybridizing in the helix 18 bulge between positions 447 and 454 resulted in significant current output over background. However, when the junction of probe pairs was in the double-stranded region of helix 18, little or no current output signal was detectable. These results demonstrate the importance of non-double-stranded regions in accessibility to probe binding.

The effect of probe length on signal intensity was examined. Experiments were performed at room temperature and involved a series of capture and detector probes, where both members of the probe pair were of equal lengths. In these experiments, probes ranging from 10 to 35 nucleotides in length were used; in each case, the junction between probe hybridization sites was between positions 453 and 454. Current output (in nanoamperes) was measured using a series of capture and detector probes of increasing length hybridized to 16S rRNA released from 9.1×10^6 *E. coli* cells. Increases in probe length resulted in increases in signal intensity until a length of 20 nucleotides was reached. There was a positive correlation (Spearman rank correlation [r_s] = 1.0; $P < 0.001$) between signal intensity and the length of the capture and detector probes (data not shown). Above 20 nucleotides, signal intensity was lost, presumably due to the formation of a potential hairpin loop structure in the longer probes at ambient temperature. Previous sensor studies had been conducted with 35-mer detector and capture probes, which had performed well using a hybridization temperature of 65°C (10), which would be unfavorable for the formation of the hairpin loop structure. These results demonstrate the relationship between probe length and signal intensity and the importance of using shorter probes for hybridization at ambient temperature.

When capture probes of various lengths were paired with a 13-mer detector probe, a twofold increase in signal intensity

TABLE 1. Sequences of oligonucleotide probes used in this work

Probe ^a	Designation (length) ^b	Sequence (5'-3')
<i>E. coli</i> probe pairs		
Capture	EC442C (8-mer)	5'-CTCCCCGC-3'
Detector	EC429D (13-mer)	5'-TGAAAGTACTTTA-3'
Capture	EC444C (10-mer)	5'-CTTCCTCCCC-3'
Detector	EC433D (11-mer)	5'-GCTGAAAGTAC-3'
Capture	EC446C (10-mer)	5'-CCCTTCCTCC-3'
Detector	EC437D (9-mer)	5'-CCGCTGAAC-3'
Capture	EC447C (10-mer)	5'-TCCCTTCCTC-3'
Detector	EC438D (9-mer)	5'-CCCGCTGAA-3'
Capture	EC448C (10-mer)	5'-CTCCCTTCCT-3'
Detector	EC440D (8-mer)	5'-CCCCGCTG-3'
Capture	EC449C (10-mer)	5'-ACTCCCTTCC-3'
Detector	EC441D (8-mer)	5'-TCCCCGCT-3'
Capture	EC451C (12-mer)	5'-CTTTACTCCCTT-3'
Detector	EC443D (8-mer)	5'-CCTCCCCG-3'
Capture	EC453C (11-mer)	5'-ACTTTACTCCC-3'
Detector	EC444D (9-mer)	5'-TTCCTCCCC-3'
Capture	EC454C (12-mer)	5'-TAACTTTACTCC-3'
Detector	EC444D (10-mer)	5'-CTTCCTCCCC-3'
Capture	EC455C (13-mer)	5'-ATTAACCTTTACTC-3'
Detector	EC445D (10-mer)	5'-CCTTCCTCCC-3'
Capture	EC456C (14-mer)	5'-GTATTAACTTTACT-3'
Detector	EC446D (10-mer)	5'-CCCTTCCTCC-3'
Capture	EC458C (14-mer)	5'-AGGTATTAACTTTA-3'
Detector	EC448D (10-mer)	5'-CTCCCTTCCT-3'
Capture	EC460C (13-mer)	5'-AAGGTATTAACTT-3'
Detector	EC449D (11-mer)	5'-TACTCCCTTCC-3'
Capture	EC454C (10-mer)	5'-ACTTTACTCC-3'
Detector	EC444D (10-mer)	5'-CTTCCTCCCC-3'
Capture	EC454C (11-mer)	5'-AACTTTACTCC-3'
Detector	EC443D (11-mer)	5'-CTTCCTCCCCG-3'
Capture	EC454C (12-mer)	5'-TAACTTTACTCC-3'
Detector	EC442D (12-mer)	5'-CTTCCTCCCCGC-3'
Capture	EC454C (13-mer)	5'-TTAACTTTACTCC-3'
Detector	EC441D (13-mer)	5'-CTTCCTCCCCGCT-3'
Capture	EC454C (14-mer)	5'-ATTAACCTTTACTCC-3'
Detector	EC440D (14-mer)	5'-CTTCCTCCCCGCTG-3'
*Capture	EC454C (15-mer)	5'-TATTAACCTTTACTCC-3'
*Detector	EC439D (15-mer)	5'-CTTCCTCCCCGCTGA-3'
Capture	EC454C (20-mer)	5'-AAAGGTATTAACTTTACTCC-3'
Detector	EC434D (20-mer)	5'-CTTCCTCCCCGCTGAAAGTA-3'
Capture	EC454C (25-mer)	5'-TGAGCAAAGGTATTAACTTTACTCC-3'
Detector	EC429D (25-mer)	5'-CTTCCTGAAAGTACTTTACAACCC-3'
Capture	EC454C (30-mer)	5'-GTCAATGAGCAAAGGTATTAACTTTACTCC-3'
Detector	EC424D (30-mer)	5'-CTTCCTGAAAGTACTTTACAACCCGAAGG-3'

Continued on following page

TABLE 1—Continued

Probe ^a	Designation (length) ^b	Sequence (5'–3')
Capture Detector	EC454C (35-mer) EC419D (35-mer)	5'-TGAGCGTCAATGAGCAAAGGTATTAACCTTTACTCC-3' 5'-CTTCCCTGAAAGTACTTTACAACCCGAAGGCCTTC-3'
<i>P. mirabilis</i> probe pairs		
Capture Detector	PM449C (12-mer) PM437D (12-mer)	5'-TTATCACCTTCC-3' 5'-TCCCCGCTGAAA-3'
Capture Detector	PM450C (12-mer) PM438D (12-mer)	5'-CTTATCACCTTC-3' 5'-CTCCCCGCTGAA-3'
Capture Detector	PM451C (12-mer) PM439D (12-mer)	5'-CCTTATCACCTT-3' 5'-CCTCCCCGCTGA-3'
Capture Detector	PM452C (11-mer) PM441D (11-mer)	5'-CCTTATCACCT-3' 5'-TCCTCCCCGCT-3'
Capture Detector	PM453C (12-mer) PM441D (12-mer)	5'-AACCTTATCAC-3' 5'-TTCCTCCCCGCT-3'
Capture Detector	PM454C (13-mer) PM441D (13-mer)	5'-TTAACCTTATCAC-3' 5'-CTTCCTCCCCGCT-3'
Capture Detector	PM455C (13-mer) PM442D (13-mer)	5'-ATTAACCTTATCA-3' 5'-CCTTCCTCCCCGC-3'
*Capture *Detector	PM210C (21-mer) PM189D (21-mer)	5'-CATCCGATAGTGCAAGGTCCG-3' 5'-AAGAGCCCCTGCTTTGGTCCG-3'
<i>K. pneumoniae</i> probe pairs		
Capture Detector	KP449C (9-mer) KP440D (9-mer)	5'-(ACT)CGCCTTCC-3' 5'-TCCCCGCTG-3'
Capture Detector	KP450C (11-mer) KP439D (11-mer)	5'-T(TC)A(ACT)CGCCTTC-3' 5'-CTCCCCGCTGA-3'
*Capture *Detector	KP451C (11-mer) KP440D (11-mer)	5'-CT(TC)A(ACT)CGCCTT-3' 5'-CCTCCCCGCTG-3'
Capture Detector	KP452C (10-mer) KP442D (10-mer)	5'-CT(TC)A(ACT)CGCCT-3' 5'-TCCTCCCCGC-3'
Capture Detector	KP453C (10-mer) KP443D (10-mer)	5'-CCT(TC)A(ACT)CGCC-3' 5'-TTCCTCCCCG-3'
Capture Detector	KP454C (11-mer) KP443D (11-mer)	5'-AACCT(TC)A(ACT)CGC-3' 5'-CTTCCTCCCCG-3'
Capture Detector	KP455C (12-mer) KP443D (12-mer)	5'-TTAACCT(TC)A(ACT)CG-3' 5'-CCTTCCTCCCCG-3'
<i>P. aeruginosa</i> probe pairs		
Capture Detector	PA132C (11-mer) PA122D (11-mer)	5'-CTTTCTCCCTC-3' 5'-AGGACGTATGC-3'
Capture Detector	PA132C (12-mer) PA120D (12-mer)	5'-ACTTTCTCCCTC-3' 5'-AGGACGTATGCG-3'
Capture Detector	PA132C (13-mer) PA119D (13-mer)	5'-CACTTTCTCCCTC-3' 5'-AGGACGTATGCGG-3'
*Capture *Detector	PA132C (15-mer) PA117D (15-mer)	5'-CCCACTTTCTCCCTC-3' 5'-AGGACGTATGCGGTA-3'
Capture Detector	PA132C (20-mer) PA112D (20-mer)	5'-GATCCCCCACTTTCTCCCTC-3' 5'-AGGACGTATGCGGTATTAGC-3'

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TABLE 1—Continued

Probe ^a	Designation (length) ^b	Sequence (5'–3')
Capture	PA849C (15-mer)	5'-GCCACTAAGATCTCA-3'
Detector	PA834D (15-mer)	5'-AGGATCCCAACGGCT-3'
Capture	PA849C (20-mer)	5'-GCTGCGCCACTAAGATCTCA-3'
Detector	PA829D (20-mer)	5'-AGGATCCCAACGGCTAGTCG-3'
<i>Enterococcus</i> sp. probe pair		
*Capture	EF220C (20-mer)	5'-ACCGCGGGTCCATCCATCAG-3'
*Detector	EF200D (20-mer)	5'-CGACACCCGAAAGCGCCTTT-3'
<i>Enterobacteriaceae</i> probe pair		
*Capture	EB1275C (23-mer)	5'-ACTTTATGAGGTCCGCTTGCTCT-3'
*Detector	EB1252D (23-mer)	5'-CGCGAGGTCGCCTTCCTTTGTAT-3'
Universal bacterial probe pair		
*Capture	UNI795C (19-mer)	5'-ACGGCGTGGACTACCAGGG-3'
*Detector	UNI776D (19-mer)	5'-TATCTAATCCTGTTTGTC-3'

^a Probes used in the 16-sensor array are indicated by asterisks.
^b Each probe designation contains the first letters of the genus and species names of the bacterium for which the probe is designed (or another abbreviation; see text), the position of the first nucleotide in the probe hybridization location (*E. coli* 16S rRNA numbering), and “C” for a capture probe or “D” for a detector probe. The designations are followed by the probe lengths (in parentheses).

was observed for 10-mer versus 20-mer capture probes. In contrast, when detector probes of various lengths were paired with a 13-mer capture probe, no effect on signal intensity was observed. Current output (in nanoamperes) was measured using a series of capture and detector probes hybridized to 16S

rRNA released from 9.1×10^6 *E. coli* cells. In both sets of experiments, the junction between the capture and detector probe hybridization sites was located between nucleotides 453 and 454. There was a positive rank correlation ($r_s = 0.93$; $P < 0.01$) between signal intensity and capture probe length, and a

FIG. 1. Electrochemical signal intensity as a function of probe hybridization location. (A) Means and SDs from experiments performed in duplicate demonstrating that signal intensity of current (–nA) is dependent on the first nucleotide of the capture probe hybridization site being located in the bulge between positions 447 and 454 of 16S rRNA helix 18. (B) Structure of *E. coli* 16S rRNA helix 18 and the nucleotide positions in the bulge.

FIG. 2. Sensitivity of the electrochemical sensor assay. Means and SDs from *E. coli* dilution experiments performed in duplicate are shown; the dashed horizontal line indicates the current output threshold for duplicate results significantly greater than those for the negative control ($P < 0.01$).

borderline negative correlation ($r_s = -0.67$; two-sided P value of 0.11) between signal intensity and detector probe length (data not shown).

These results demonstrate that the effect of probe length on

signal intensity was primarily a function of capture probe length.

Specificity was affected by probe length. When probe pairs of various lengths designed to hybridize to *E. coli* helix 18 were examined for specificity using a variety of bacteria, it was found that 20-mer probes had significant cross-reactivity with *P. aeruginosa* (data not shown). For this reason, further studies were performed with *E. coli*-specific capture and detector probes of 15 nucleotides in length; capture probe EC454C (15-mer) was paired with detector probe EC439D (15-mer). Experiments involving fivefold dilutions of *E. coli* cells showed that the 15-mer capture and detector probes had a sensitivity threshold of 330 cells (Fig. 2). This level of detection sensitivity is similar to that of 35-mer capture and detector probes with a 65°C hybridization temperature (10).

Ambient-temperature hybridization using relatively short probes was able to distinguish between organisms having nearly identical sequences. Figure 3A shows that the *K. pneumoniae* and *P. mirabilis* 16S rRNA sequences differ by only a single nucleotide at position 455 (numbering based on the *E. coli* 16S rRNA sequence). Experiments were performed using a series of capture and detector probe pairs with hybridization site junctions near position 455. Each pair of *K. pneumoniae*-

FIG. 3. Discrimination of a 16S rRNA single-nucleotide polymorphism as a function of probe hybridization location. (A) 16S rRNA sequences of *Proteus mirabilis* and *K. pneumoniae* and the hybridization locations of capture and detector probes. (B) Mean current output (in nanoamperes) and SD from experiments performed in duplicate, with the nucleotide location at the junction of the capture and detector probe hybridization sites given on the horizontal axis. NC, negative control.

FIG. 4. Specificity of the electrochemical sensor array. Sensor arrays (see text for details) were tested with six uropathogen species. Means and SDs of log signal intensities of current ($-nA$) from duplicate sensors are plotted on the vertical axes in an antilog scale. Dashed horizontal lines indicate the current output threshold for duplicate results significantly greater than those for the negative control (NC) ($P < 0.001$). EC, *E. coli* probe; KP, *K. pneumoniae* probe; PM, *P. mirabilis* probe; PA, *P. aeruginosa* probe; EF, *E. faecalis* probe; UNI, universal bacterial probe; EB, probe able to detect all members of the family *Enterobacteriaceae*.

specific probes produced signals with *K. pneumoniae* 16S rRNA as the target that were significantly greater than those with *P. mirabilis* 16S rRNA. Likewise, *P. mirabilis* 16S rRNA yielded higher signals for *P. mirabilis*-specific probes than did *K. pneumoniae* 16S rRNA (data not shown). These results demonstrate the exquisite specificity achievable using shorter probes and ambient-temperature hybridization.

A 16-sensor array was validated for hybridization at ambient temperature by use of a panel of well-characterized bacterial strains obtained from the ATCC. Figure 4 shows the results for sensor arrays functionalized in duplicate with the following

capture probe types (specific for the organisms listed in parentheses): EC (*E. coli*), KP (*K. pneumoniae*), PM (*P. mirabilis*), PA (*P. aeruginosa*), and EF (*E. faecalis*). Also included are results for a universal bacterial probe (UNI) and a probe able to detect all members of the family *Enterobacteriaceae* (EB). Table 1 and Fig. 5 show probe pair sequences. Two negative-control sensors in each array were functionalized with the universal capture probe and exposed to the universal detector probe but no bacterial lysate. As shown in Fig. 4, the sensor array correctly identified all five target organisms and allowed the differentiation of *K. pneumoniae* from *Enterobacter* species.

FIG. 5. Alignment of pathogen 16S rRNA sequences. The positions of unique nucleotides (red) and sites of detector (gray background) and capture (gold background) probe hybridization are shown for *P. mirabilis* and *E. faecalis* (A) and *K. pneumoniae* and *E. coli* (B).

In previous studies, it had not been possible to design a probe pair able to distinguish between *Klebsiella* and *Enterobacter* species (9, 10). Weak cross-reactivity of the *E. coli* probes with *P. aeruginosa* 16S and of the *P. aeruginosa* probes for *P. mirabilis* 16S was easily differentiated from specific reactions by comparison of the species-specific signal with that of the universal probe. The data shown in Fig. 4 are quite comparable to results obtained using the same 16-sensor array to test a number of specimens from patients with urinary tract infections (data not shown), indicating that the electrochemical sensor assay using shorter probes and ambient-temperature hybridization is compatible with clinical urine specimens, as shown for earlier probe and temperature schemes (9). These results demonstrate the utility of using combinations of probe pairs in an electrochemical sensor array.

DISCUSSION

We have previously described an electrochemical DNA sensor for bacterial detection that relies on 65°C hybridization of 35-mer oligonucleotide probes with the 16S component of rRNA (7, 9, 10, 17). In those studies, we did not examine the dependence of electrochemical signal intensity and assay sensitivity on probe length and hybridization location, which have previously been shown to be key determinants of the detection sensitivity threshold (6). In addition, the extent to which hybridization temperature played a role in the denaturation of the 16S rRNA target sequences and their accessibility to probe binding was unclear. In this study, we found that capture and detector probes as short as 8 nucleotides in length could be used to detect bacterial pathogens by use of an assay conducted entirely at ambient temperatures (20 to 25°C). There appeared to be no sensitivity benefit to using a higher hybridization temperature: for probe pairs binding to helix 18 of 16S rRNA, the lower limit of detection for the ambient-temperature assay was 330 *E. coli* cells, which is virtually the same as the 280-*E. coli*-cell detection limit observed when a hybridization temperature of 65°C was used (10). The ambient-temperature assay was found to have an important specificity advantage in being able to differentiate species-specific target sequences differing by only a single nucleotide. These studies led to the development of a panel of species-specific probe

pairs with lengths in the range of 11 to 23 nucleotides for use on a sensor array chip that was demonstrated to be able to identify the most common uropathogens.

Because of their high copy number in bacterial cells and sequence stability within phylogenetic groups, ribosomal subunits are commonly targeted by detection methods using species-specific oligonucleotide probes. For example, fluorescence in situ hybridization (FISH) typically involves oligonucleotide probes targeting 16S rRNA (11). FISH probes tagged with various fluorescent dyes can be used to simultaneously visualize and differentiate multiple types of organisms within mixed populations of microbial cells (18). In FISH, probe specificity is achieved by adjustment of the formamide concentration in the hybridization buffer and use of a hybridization temperature of between 37 and 50°C. In addition to improving probe specificity, these hybridization conditions may contribute to the exposure of target sequences by destabilizing the structure of the highly folded 16S rRNA target molecule. Our challenge was to identify capture and detector probe pairs that work in tandem and bind efficiently to accessible target sequences without the need to use heat or chemical methods to denature the 16S rRNA target molecule. Our hybridization solution had to be buffered at a neutral pH to prevent denaturation of the streptavidin anchoring the biotinylated capture probe to the sensor surface. Despite the use of these “native” hybridization conditions, the sensitivity limit of 330 *E. coli* cells for the ambient-temperature electrochemical sensor assay that we observed (Fig. 2) was virtually the same as that reported previously using a hybridization temperature of 65°C (10). This is the first study that demonstrates binding of oligonucleotide probes to 16S rRNA at ambient temperature without denaturing chemicals in the hybridization buffer.

Our studies focused on helix 18 of the 16S bacterial ribosome. Because of its high interspecies sequence diversity, helix 18 is a useful target region for probes designed to differentiate bacterial species. As shown in Fig. 1B, helix 18 consists of two double-stranded regions separated by a bulge between positions 447 and 454. We examined the electrochemical signal intensity by use of a series of probes with comparable melting temperatures to determine the optimal capture/detector probe pair configuration relative to the structure of helix 18. We

found that signal intensity was high for detector/capture probe combinations where the detector probe hybridized to both the proximal double-stranded region and the bulge, while the capture probe hybridized to both the bulge and the distal double-stranded region. Probe pairs in which either the detector probe or the capture probe did not hybridize to the bulge yielded a significantly lower electrochemical signal. These results confirm and extend a prior FISH study that systematically mapped the accessibility of the entire 16S ribosome of *Escherichia coli* by use of 18-mer oligonucleotide probes (6). In the FISH study, probe Eco440, which produced one of the highest fluorescence signal intensities of all the probes tested, hybridized to the entire bulge and the proximal double-stranded region of helix 18. Although the FISH study used single oligonucleotide probes and our sensor studies use a probe pair system, the results are consistent and together help to define the accessibility of helix 18 to oligonucleotide probe binding.

An important advantage of using shorter oligonucleotide probes with the electrochemical sensor is the ability to differentiate organisms with single-nucleotide differences in the 16S rRNA sequence. While most uropathogens have multiple sequence differences in the target hybridization region of helix 18, *K. pneumoniae* and *P. mirabilis* differ by only a single nucleotide at position 455 (Fig. 3A). We demonstrated that a series of probe pairs involving a *K. pneumoniae*-specific capture probe were all able to bind preferentially to *K. pneumoniae*. There was virtually no detectable signal from *P. mirabilis* 16S rRNA when position 455 was near the junction between the capture and detector. This result suggests that base pair stacking during target hybridization may be important in the interaction of the capture and detector probes and is consistent with our previously reported finding of higher signal intensity for probe pairs that lacked a gap between the capture and detection hybridization sites (10). As shown in Fig. 4, the *K. pneumoniae*-specific KP451C/KP440D capture/detector probe pair was able to differentiate not only between *K. pneumoniae* and *P. mirabilis* but also between *K. pneumoniae* and *E. aerogenes*. Using our previous system of 35-mer probes hybridizing at 65°C, we had not been able to design a capture/detector probe pair that was able to differentiate between *Klebsiella* and *Enterobacter* species (9, 10). The development of highly species-specific probe pairs is also of great interest with respect to future prospects for the analysis of polymicrobial specimens. The results in Fig. 4 suggest that detailed comparison of the complete pattern of species-specific signal intensities to the intensity of the universal probe pair may provide sufficient information to detect the presence of two or more organisms in the same sample, as long as both organisms are present at comparable magnitudes.

The results in Fig. 3B indicate that there can be a tradeoff between signal intensity and cross-hybridization. Higher signals for *K. pneumoniae* 16S rRNA were obtained for capture probes with hybridization sites beginning at positions 449 to 451 than for those beginning at positions 442 to 455. However, the former probes also bound weakly to *P. mirabilis* 16S rRNA. We chose capture probe KP451C for the sensor array because of the higher signal, and presumably higher sensitivity, for *K. pneumoniae* 16S rRNA, because we knew that the array would also include a *P. mirabilis*-specific capture probe. The results in Fig. 4 demonstrate that, despite the limited cross-reactivity between the *K. pneumoniae*-specific capture probe and *P.*

mirabilis 16S rRNA, there was unambiguous identification of *P. mirabilis* when the results were read in the context of the entire electrochemical sensor array.

In summary, there is an urgent need to develop point-of-care technologies that would improve health care delivery through rapid, portable diagnostic testing. Electrochemical sensors have tremendous potential for point-of-care testing because of the capacity for miniaturization of their components. Most molecular systems for the detection of microbial pathogens, including PCR, require temperature control systems that are typically bulky and have high power requirements. Elimination of the need for a temperature control system would greatly simplify electrochemical sensor design and fabrication. Before widespread clinical application of this technology can begin, additional refinements are needed to improve sensitivity and specificity and to discriminate among a wide range of gram-negative and gram-positive uropathogens. Nevertheless, the studies presented here demonstrate the feasibility of using short oligonucleotide probes for electrochemical detection of uropathogens at ambient temperature and represent a significant accomplishment toward our long-term goal of developing a hand-held device for rapid bacterial pathogen detection.

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