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Quenched autoligation probes allow discrimination of live bacterial species by single nucleotide differences in rRNA

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ABSTRACT

Quenched autoligation (QUAL) probes are a class of self-reacting nucleic acid probes that give strong fluorescence signal in the presence of fully complementary RNAs and selectivity against single nucleotide differences in solution. Here, we describe experiments designed to test whether QUAL probes can discriminate between bacterial species by the detection of small differences in their 16S rRNA sequences. Probes were introduced into live cells using small amounts of detergent, thus eliminating the need for fixation, and fluorescence signal was monitored both by microscopy and by flow cytometry without any washing steps. The effects of probe length, modified backbone, probe concentration and growth state of the bacteria were investigated. The data demonstrate specific fluorescence discrimination between three closely related bacteria, *Escherichia coli*, *Salmonella enterica* and *Pseudomonas putida*, based on single nucleotide differences in their 16S rRNA. Discrimination was possible with cells in mid-log phase or in lag phase. These results suggest that QUAL probes may be useful for rapid identification of microorganisms in laboratory and clinical settings.

INTRODUCTION

There is a great deal of interest in rapid and highly accurate detection of microorganisms for applications in molecular diagnostics, clinical chemistry, molecular biology, environmental sampling and food monitoring. Ribosome-targeted nucleic acid hybridization probes have been commonly applied for the identification of prokaryotic organisms in environmental (1–3) and clinical samples (4,5). rRNA is an attractive target in bacterial cells because of its abundance, its accessibility (6,7) and the availability of sequences for a wide range of organisms (8,9). Furthermore, there is enough sequence variability that family-specific and sometimes even genus- or species-specific rRNA-targeted probes can be used to help classify bacterial samples (3). However, closely related bacteria, such as *Escherichia coli* and *Salmonella* species, have highly conserved rRNA sequences that are extremely difficult or impossible to distinguish using standard hybridization-based methods (10,11). Moreover, simple hybridization probes cannot be applied in intact cells because of the requirement for washing away of unbound probes; it would be advantageous to use live cells because this would save time-consuming and potentially error-prone fixation and washing steps, as well as potentially allowing studies on the dynamics and localization of RNA. For these reasons there has been considerable interest in the development of more highly specific probes that rely on a sequence-directed conformation change or chemical reaction to yield signals.

Several new strategies for oligonucleotide-based probes that can detect sequences with high specificity have been reported recently, including a Staudinger reaction approach (12), target-assisted self-cleavage probes (13), PNA-based hybridization assays (14,15), metal-mediated DNA cleavage (16), molecular beacons (17,18) and quenched autoligation (QUAL) probes (19–21). Of these methods, only molecular beacons and QUAL probes have been thus far applied to cells. Early studies have reported the targeting of rRNA in live *E.coli* with QUAL probes (22), while no reports yet exist on the testing of ribosome-targeted molecular beacon probes in live bacteria. Furthermore, there are no reports of molecular beacons distinguishing rRNA single nucleotide polymorphisms (SNPs) in live or fixed bacterial cells.

The goal of this work is rapid and accurate discrimination of live bacteria of different species by fluorescence that can be observed simply by flow cytometry or fluorescence microscopy. The use of very short probes that react with one another

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Dedicated to Professor Peter Dervan on his 60th birthday.

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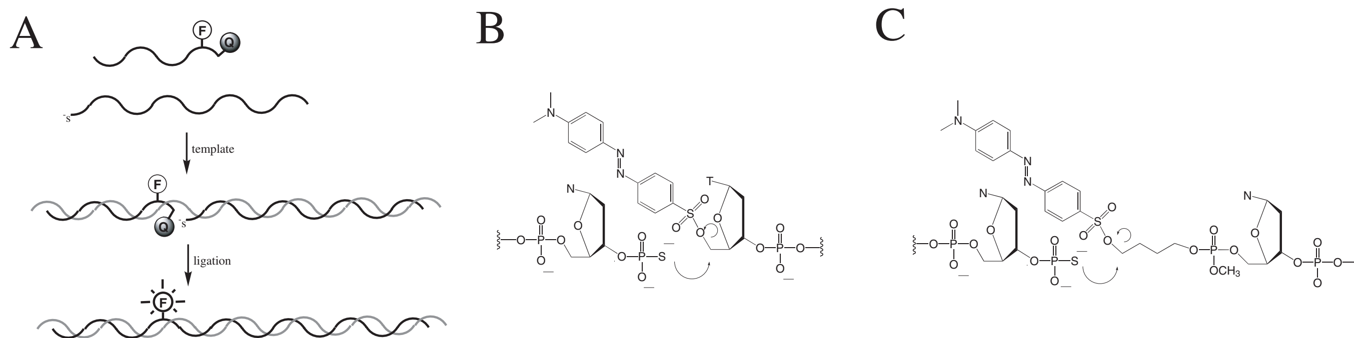

Figure 1. Structure and mechanism of QUAL probes. (**A**) Diagram of ligation reaction on complementary template. (**B**) Molecular mechanism of ligation reaction using earlier dabsyl-dT electrophile. (**C**) Molecular mechanism of ligation reaction using the present universal dabsyl linker.

is a strategy developed for enhancing sequence discrimination while yielding a signal change that might be applied in intact cells (21). QUAL probes consist of a pair of modified oligonucleotides: an electrophilic probe containing an internal fluorophore and a dabsyl quencher attached to the 5′-terminus by a sulfonate ester linkage, and a nucleophilic probe containing a 3′-phosphorothioate group. In the presence of target, the two probes bind side-by-side, and nucleophilic displacement of the quencher by the phosphorothioate leads to probe ligation and unquenching of the fluorophore (Figure 1A) (21). The reaction can be monitored by the gradual increase in fluorescence signal appearing over minutes to hours. In most previous work, the dabsyl quencher was attached to the 5′-hydroxyl of a terminal thymidine (Figure 1B). Recently, an 'universal linker' for attachment of the quencher to DNA (Figure 1C) was reported (23); this allowed application to generalized targeting of sequences without restriction. Furthermore, the linker forms a bulge in the ligated DNA, thereby destabilizing the duplex, which yielded amplification of up to 92 signals per template in one study (23).

In early experiments with fixed *E.coli* cells, 16S rRNA-targeted QUAL probes using dabsyl-dT showed substantial signal only when the electrophilic and nucleophilic probes bound adjacently, and none when targeted to sites several hundred nucleotides apart (24). Preliminary reports suggested that QUAL probes could also be used for generating signals in living bacteria (22). However, it was unknown whether different bacterial species could be targeted with such probes; this is an issue because of possible differences in cell wall structure, intracellular properties and RNA sequence. Nor was it known whether single nucleotide differences could be discriminated in live cells. The preferred probe lengths were not established, and no data existed on whether altering the probe backbone to render it more resistant to degradation would improve signal.

In this work we addressed these issues experimentally, and investigated whether optimized QUAL probes with the universal dabsyl linker are able to discriminate single nucleotide differences in the 16S rRNA sequences of three closely related bacterial species: *E.coli*, *Salmonella enterica* and *Pseudomonas putida*. When the intact cells were monitored by using flow cytometry or fluorescence microscopy, significant fluorescence signals were observed only when fully matching probes were used; in the presence of probes with as little as a single mismatch, significantly diminished signals were observed, suggesting the application of such probes in bacterial identification.

MATERIALS AND METHODS

QUAL probes

Universal butanediol linker phosphoramidite was synthesized as described previously (23). DNA and 2′-OMe RNA probes were prepared using literature methods (21,23). Reagents and standard phosphoramidites for DNA synthesis were obtained from Applied Biosystems. Ultramild phosphoramidites and CPG columns, 3′-phosphate CPG columns, sulfurizing reagent and fluorescein-dT phosphoramidite were obtained from Glen Research. Oligonucleotides containing a 3′-phosphorothioate were prepared using 3′-phosphate CPG columns and sulfurizing reagent as described previously (20). 3′-Phosphorothioate and helper oligonucleotides were deprotected using ammonium hydroxide at 55°C for 16–20 h and were used without further purification. Dabsyl- and fluorescein-containing oligonucleotides were prepared using Ultramild phosphoramidites to allow for mild deprotection at room temperature in methanolic 0.05 M K_2CO_3 for 4–6 h. Fluorescein was introduced using the fluorescein-dT phosphoramidite. After deprotection, dabsyl probes were purified by high-performance liquid chromatography using a gradient of acetonitrile in 0.1 M TEAA buffer (5–65% over 45 min). Purified probes were stored at −80°C. All oligonucleotide sequences are listed in Supplementary Material.

Thermal denaturation studies

RNA templates were purchased from Integrated DNA Technologies. Solutions for melting temperature (T_m) determination contained 3 μM each of template and complementary or mismatched strands in 6× SSC buffer at pH 7.0. The solutions were heated to 80°C for at least 15 min to denature any duplexes, and then cooled at 0.5°C/min in the UV-vis spectrophotometer Varian Cary 1 equipped with a thermoprogrammer. The melting studies were carried out in Teflon-stoppered 1 cm path length quartz cuvettes under a steady flow of nitrogen to prevent condensation on the quartz wall at low temperature. Absorbance was monitored at 260 nm. T_m values were generated by computer fitting of the data with MeltWin software.

Bacteria culture and preparation

Materials and reagents were sterilized by autoclaving at 120°C for 20 min. Buffers were prepared using RNase-free water. *E.coli* K12 (ATCC 10798) and *S.enterica* (ATCC 700720) were grown to mid-log phase (OD_{600} = 0.4–0.6) or lag phase (OD_{600} = 1.2–1.4) in Luria–Bertani (LB) media (DIFCO) at 37°C with rapid shaking. *P.putida* (ATCC 49128) were grown in Nutrient Broth (DIFCO) with rapid shaking at 37°C. Aliquots of media were centrifuged for 5 min at 9000 *g* supernatant was removed and the pellets were washed with 0.5 ml of phosphate-buffered saline buffer (pH 7.2). The pellets were then resuspended in hybridization buffer [6× SSC buffer (pH 7.0) containing 0.05% SDS].

In situ ligation reactions

Aliquots of bacteria suspended in hybridization buffer (100 μl) were treated with dabsyl probe as described (typically 200 nM), phosphorothioate probe (2 μM) and helper probes (3 μM each). The reactions were incubated in the dark at 25 or 37°C for 2 h, then monitored by microscopy or flow cytometry without any washing steps. Judging by microscopy and flow cytometry (forward and orthogonal scattering), cellular morphology was not affected by the addition of QUAL probes.

Fluorescence microscopy

Slides were prepared by sandwiching a solution of 1% agarose between two glass slides. One slide was removed after the agarose solidified, then 3 μl of bacteria in hybridization buffer was directly spotted onto the agarose and covered with a glass coverslip. Fluorescence images were obtained with an epifluorescence microscope (Nikon Eclipse E800 equipped with ×100 objective Pan Fluor apo) with super high-pressure mercury lamp (Nikon model HB-10103AF), excitation 460–500 nm, using a SPOT RT digital camera and SPOT Advanced imaging software. Typical digital camera settings were as follows: exposure time green 3 s, no binning, gain = 2.

Flow cytometry

Flow cytometry data were collected on a FACScan instrument (Becton Dickinson) using an argon laser (ex = 488 nm). In a typical sample, 50 000 events were recorded. Data were analyzed using FlowJo software version 4.6.1 (Tree Star, Inc.).

RESULTS

Probe design

16S rRNA sequences for *E.coli*, *S.enterica* and *P.putida* were obtained from the NCBI Entrez Genome Project (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Sequence alignments were performed using ClustalW (25). Each of these bacteria species has seven 16S rRNA operons (26), and sequence alignment showed the presence of several sequence differences between the individual operons. These variable positions were avoided when designing probes for bacteria discrimination. Sequence alignments between the three bacterial species showed >97% sequence identity between *E.coli* and *S.enterica* and ~85% sequence identity between *E.coli* and *P.putida*.

To test whether QUAL probes could discriminate very small sequence differences, we chose target sites where a single mismatch, or small number of mismatches, was present between the different bacterial species. Target sites were also selected based on their accessibility in *E.coli* to fluorescence *in situ* hybridization probes (6). In addition, sequences were chosen to have an adenine a few bases past the mismatch for incorporation of fluorescein-dT into the dabsyl probe. The probes were designed so that the mismatch would occur at the center of the dabsyl probe, which yields highest selectivity for autoligating probes (19). Dabsyl oligonucleotides were typically short (8 or 9 nt in length, but varied in different experiments), containing the dabsyl group attached to the 5′-terminus by a butanediol linker (Figure 1C) and fluorescein-dT 2–3 bases from the linker. The nucleophilic phosphorothioate probes were designed to bind to the target RNA adjacent to the dabsyl probe, and since they were not required to sense small nucleotide differences, we used longer 12mer to 15mer to increase binding. In addition to the dabsyl and phosphorothioate probes, we used 'helper' oligonucleotides (18 nt), designed to bind next to the ligating probe pairs to help disrupt secondary structure of the rRNA, thereby increasing target accessibility (27).

Optimization of conditions for discrimination of *E.coli* and *S.enterica*

QUAL probes were introduced into the bacterial cells by using 0.05% SDS in 6× SSC buffer. After 2 h incubation under these conditions, bacteria remained viable, as they continued to grow when resuspended in LB media. In order to optimize conditions for using QUAL probes to discriminate live bacteria, we chose to focus on a C/A SNP at 16S ribosomal position 745 in *E.coli* and *S.enterica* (Figure 2, Ec/Se584 probes). We first addressed whether DNA or 2′-OMe RNA dabsyl probes would perform better. The 2′-OMe backbone is known to be resistant to degradation as compared with unmodified DNA, and to increase affinity for RNA (28). We prepared 9mer DNA and 2′-OMe RNA dabsyl probes specific for *E.coli* or *S.enterica* (Ec/Se584 sequence), and used them with DNA phosphorothioate and helper probes (for all sequences see Supplementary Material). These experiments revealed that 2′-OMe RNA probes gave somewhat enhanced signal as compared with the DNA probes. However, nearly no discrimination of the two bacteria species was observed (Figure 3).

A lack of sequence discrimination might be explained by a binding affinity that was too high, allowing even mismatched probes to bind strongly enough for ligation to occur. Thus the impact of varying probe length on identification of *E.coli* and *S.enterica* was then examined. We prepared a series of DNA dabsyl probes ranging from 6 to 9 bases in length with the SNP site in the center of the probes, the position of greatest selectivity for self-ligating probes (19). Bacteria were grown to mid-log phase and treated with these QUAL probes, incubated at 25 or 37°C for 2 h, then the fluorescence intensity was monitored by flow cytometry. Figure 4A–D shows that nearly no signal from ligation was observed when 6mer or 7mer dabsyl probes were used, suggesting that they were too short to yield sufficient binding affinity.

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Ec/Se584

E CCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG 778
S CCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG 778

Target Site 1

E CCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCG 898
S CCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGCCG 898
P TCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCG 877

Target Site 2

E GCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGC 868
S GCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGC 868
P GCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGC 847

Target Site 3

E CCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAG 682
S CCTGGGAACTGCATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAG 682
P CCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCT 661
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Figure 2. Sequence alignments for probe target sites. Polymorphisms are in red. Binding sites for dabsyl and phosphorothioate probes are highlighted in blue and green, respectively.

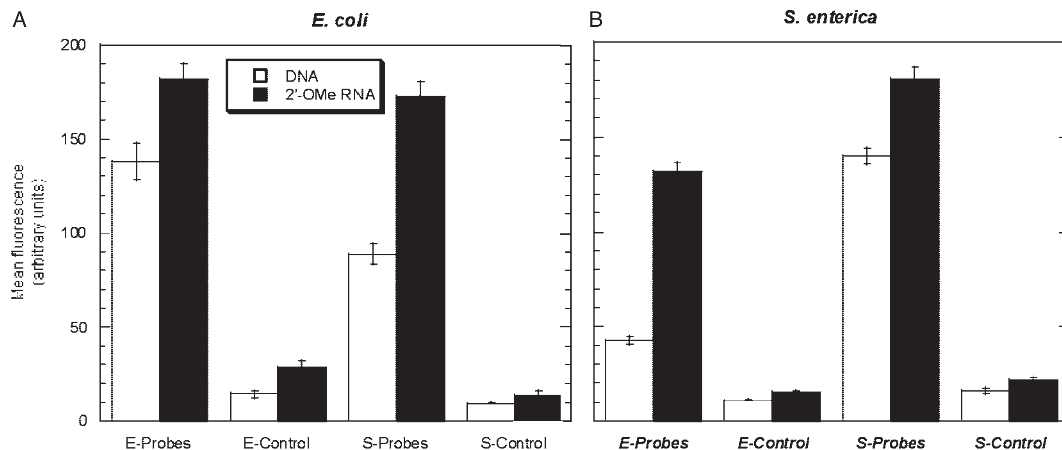

Figure 3. Comparison of DNA and 2′-OMe RNA dabsyl probes. Bacteria were incubated at 37°C with 200 nM dabsyl probe, 3 μM helper probes and 2 μM phosphorothioate probe (in cases indicated) for 2 h, then analyzed by flow cytometry. Mean fluorescence values are from three experiments. E-Probes, *E.coli*-specific dabsyl probe and phosphorothioate; E-Control, *E.coli*-specific dabsyl probe only; S-Probes, *S.enterica*-specific dabsyl probe and phosphorothioate; S-Control, *S.enterica*-specific dabsyl probe only. (**A**) *E.coli* and (**B**) *S.enterica*.

In contrast to this, 8mer and 9mer probes did yield ligation signals. At 25°C, there was approximately equal signal when *E.coli* were treated with *E.coli*-specific 8mer or 9mer probes versus *S.enterica*-specific probes (Figure 4A). However, at 37°C, there was substantially more fluorescence in *E.coli* treated with *E.coli*-specific 9mer probes (Figure 4B), showing discrimination against a single G–T mismatch. *S.enterica* treated with 8mer probes were discriminated reasonably well at 25°C (an A–T pair versus an A–C mismatch) but not as well at 37°C, while *S.enterica* treated with 9mer probes were discriminated quite well at both 25 and 37°C (Figure 4C and D). Background signals were quantified by omitting the phosphorothioate probes; presumably, the remaining non-specific signals arise from hydrolysis or degradation of the dabsyl probe. These background signals were relatively consistent across all samples, and were higher at 37°C than at 25°C.

To investigate the relationship between probe length, mismatches and signals, we then performed melting studies on the 584E and 584S probes on RNA templates. The T_m values for these probes bound to matched and mismatched templates are shown in Table 1. As expected, there was a substantial T_m difference for the *E.coli*-specific probes bound to matched versus mismatched template, representing a C–A mismatch, while the T_m difference for the *S.enterica*-specific probes, a G–T mismatch, was small. The T_m values for matching sequences shorter than the 9mer were <37°C, which could partially account for the diminished signal in the shorter probes. The lack of signal for the short probes may also be a result of increasing sequence redundancy in the 16S rRNA.

With probe length optimized for these targets, we were next interested in learning the effects of cell growth on the effectiveness of the QUAL probes. The effect of lowering the concentration of the dabsyl probe, while keeping the

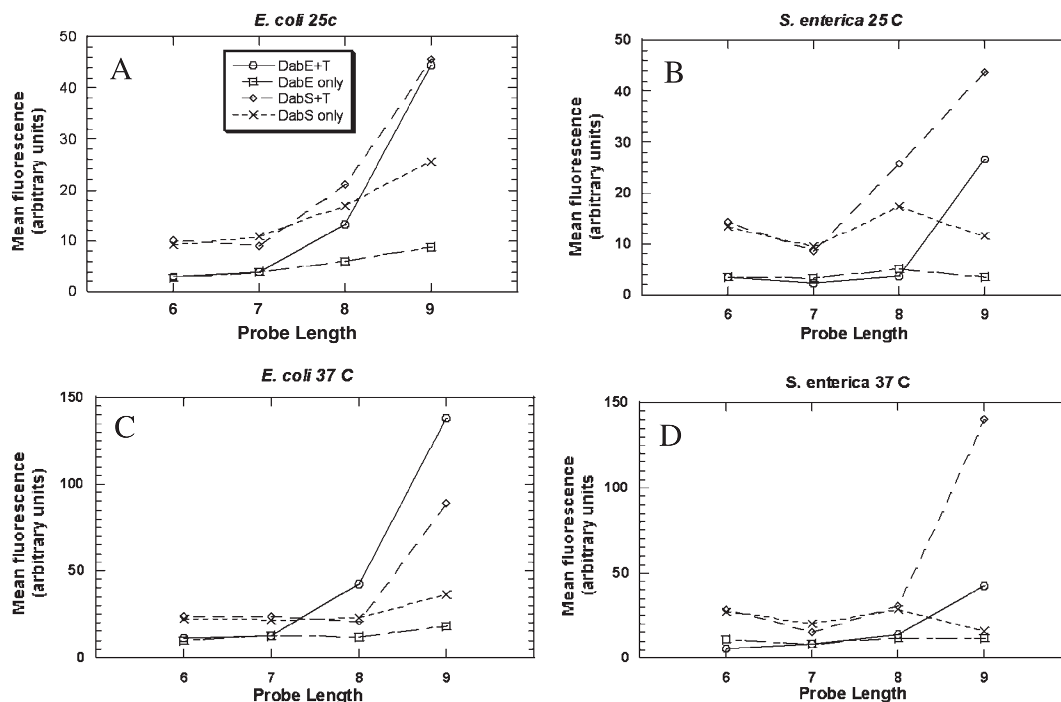

Figure 4. Comparison of dabsyl probe length on bacteria discrimination. Bacteria were incubated with 200 nM dabsyl probe, 3 µM helper probes and 2 µM phosphorothioate probe (in cases indicated) for 2 h, then analyzed by flow cytometry. Mean fluorescence values are from three experiments. (**A**) *E.coli* incubated at 25°C; (**B**) *S.enterica* incubated at 25°C; (**C**) *E.coli* incubated at 37°C; and (**D**) *S.enterica* incubated at 37°C.

Table 1. T_ms (°C) of 584E and 584S probes hybridized to RNA complementary or mismatched templates

Probe	T_m (°C) *E.coli*	*S.enterica*
E9	39	20
E8	38	19
E7	35	16
E6	28	15
S9	39	31
S8	32	24
S7	30	22
S6	24	21

concentrations of phosphorothioate and helper probes constant, was concurrently tested. Substantially less signal was observed for bacteria grown to lag phase versus those grown to log phase (Supplementary Material). Background signals when no phosphorothioate probe was added decreased when lower concentrations of dabsyl probe were used, but positive signals were also diminished. At 20 nM dabsyl probe concentration, signal was so low that nearly no fluorescence was observed when the bacteria were imaged under a fluorescence microscope. Overall, the greatest signal-to-background ratio and the discrimination were achieved when 200 nM dabsyl probes were used (Supplementary Material). Further increases in the dabsyl probe concentration did not improve discrimination, and led to lower signal-to-background ratios (data not shown).

Using the optimized conditions for mid-log phase bacteria (DNA 9mer dabsyl probe at 200 nM, 2 h incubation at 37°C), we imaged the bacteria under an epifluorescence microscope with 460–500 nm excitation (Figure 5). When dabsyl probes were added but no phosphorothioate, nearly no fluorescence was observed in these controls (Figure 5, insets). In the presence of the intended phosphorothioate probes, bright signals were observed when *E.coli* cells were treated with *E.coli*-specific dabsyl probe, and substantially less signal was observed with *S.enterica*-specific probes. Conversely, *S.enterica* treated with *S.enterica*-specific probes fluoresced brightly, and nearly no fluorescence was observed when they were treated with *E.coli*-specific probes.

Specific identification of three live bacterial strains

Encouraged by the results of the Ec/Se584 probe set, we prepared a series of probes for three new target sites, these aimed at discriminating the three types of bacteria (Figure 2). The sites were chosen based on the presence of 16S sequence differences between the three strains, and on a prediction of at least moderate accessibility (6). Although in principle each site could allow for discrimination between the three species, we chose three to test because different mismatches may yield different responses, and because accessibility in practice may differ from predicted models. For each target site, a dabsyl probe specific for each bacteria was prepared. For site 1, a single phosphorothioate probe was appropriate for all three bacteria species, but for sites 2 and 3, a different phosphorothioate specific for *P.putida* was required. We attempted to obtain T_ms for the G/T mismatch in the target site 2 probes, but were unable to determine the melting transition because of significant self-complementarity in the RNA template strand.

Bacteria grown to mid-log phase were treated with QUAL probes, incubated at 37°C for 2 h and then analyzed by flow

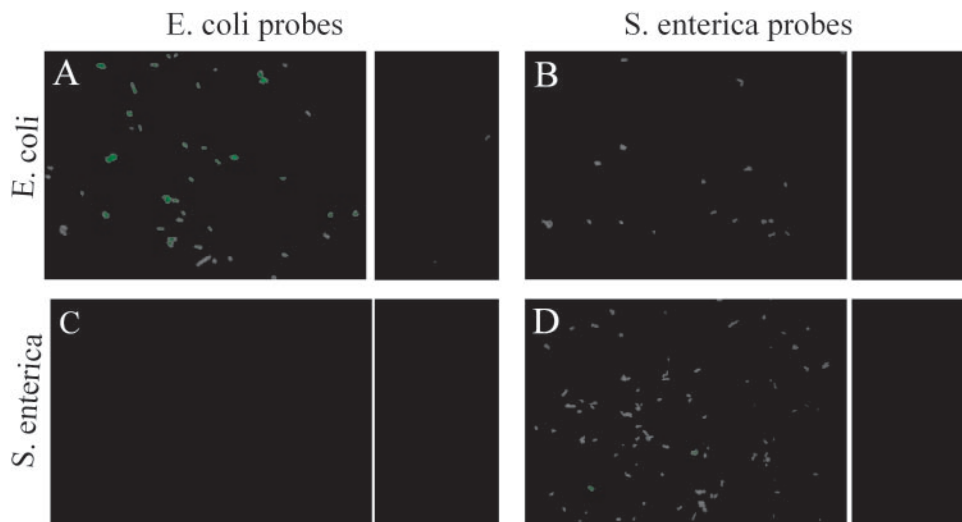

Figure 5. Fluorescence images of *E.coli* and *S.enterica* treated with Ec/Se584 probes. Bacteria were incubated with 200 nM dabsyl probe, 3 μM helper probes and 2 μM phosphorothioate probe for 2 h. Insets show images for controls when phosphorothioate probe was omitted. (**A** and **B**) *E.coli* and (**C** and **D**) *S.enterica*. (A and C) Bacteria treated with *E.coli*-specific probes are shown; and (B and D) bacteria treated with *S.enterica*-specific probes are shown.

cytometry (Figure 6). Background was measured with control samples that contained dabsyl probe but no phosphorothioate probe. For site 1, we observed substantial signal using matched probes designed for *E.coli* and *S.enterica*, while no signal over background was observed for the designed *P.putida* probes, suggesting accessibility limitations (Figure 6). In the case of site 2, signal was observed for all three bacteria when correctly matched probes were used. For site 3, no signal over background was observed for *E.coli* or *S.enterica*, while significant signal was observed for *P.putida* treated with matching probes. In all cases, little or no signal over background was observed when each of the three bacterial species was treated with probes for the other two species, indicating high sequence specificity.

Images taken with an epifluorescence microscope (Figure 7) were entirely consistent with the flow cytometry data. No signal was observed when the phosphorothioate probe was left out (images not shown), and little to no signal was observed when bacteria were treated with probes for the other two species. Images from target site 2 are shown in Figure 7, and images from sites 1 and 3 are provided in Supplementary Material. Positive signals were visible for the same probe and site combinations as seen with flow cytometry. In the case of target site 1, no signal was observed for *P.putida*, and in the case of target site 3, no signal was observed for *E.coli* or *S.enterica*. This result is the same as was seen by flow cytometry, again suggesting limited accessibility at these sites.

Overall, the results showed that probes for two 16S RNA sites gave clear discrimination of *E.coli* from *S.enterica* based on single nucleotide differences, and one site allowed discrimination of all three species. Two different analytical methods were established to document this discrimination, and both yielded identical conclusions.

DISCUSSION

The current results establish that QUAL probes can be applied in intact bacteria, allowing discrimination of single nucleotide differences among three different bacterial strains. This is markedly different from previous results with standard *in situ* hybridization methods for the detection of sequences in bacteria. The standard FISH protocol uses fluorescence-labeled oligonucleotides 15–30 nt in length. In a typical protocol, bacteria are fixed and incubated with probe, then unbound probe is removed using stringent washing conditions (2,29). Since unbound probe must be washed away to avoid background signal, the bacteria must be killed and permeabilized using paraformaldehyde or other fixative, making FISH inapplicable to live cells. Even more problematic, inefficient or inconsistent washing can lead to false positive signals. With standard FISH probes it is also extremely difficult or impossible to distinguish very small sequence differences such as SNPs (10,11).

Quenched probes obviate the need for washing away of unbound probes and thus raise the possibility of application in intact cellular specimens. The major types of quenched probes used for *in situ* hybridization experiments are molecular beacons and QUAL probes. Molecular beacons have shown promise for use in living cells, but most of these applications have focused on human cells, not bacteria (30–32). Molecular beacons have been used to discriminate different species of bacteria using fixed cells (33), but we are not aware of any cases in which they have been used to discriminate bacteria using non-fixed cells. Moreover, molecular beacons have not been shown to be able to discriminate single nucleotide differences in bacterial RNAs. A limitation with molecular beacons for *in situ* applications is non-specific signals, owing in part to binding of the molecular beacon to proteins (34,35). In the case of QUAL probes, non-specific signals can arise from spontaneous hydrolysis of the dabsyl group; however, simple omission of the nucleophile probe in a control experiment can reveal the level of this background signal. An advantage of molecular beacons is their rapid response, which can occur in real-time during PCR. Thus most bacterial applications of molecular beacons have been in real-time PCR-based assays carried out after extraction of RNAs

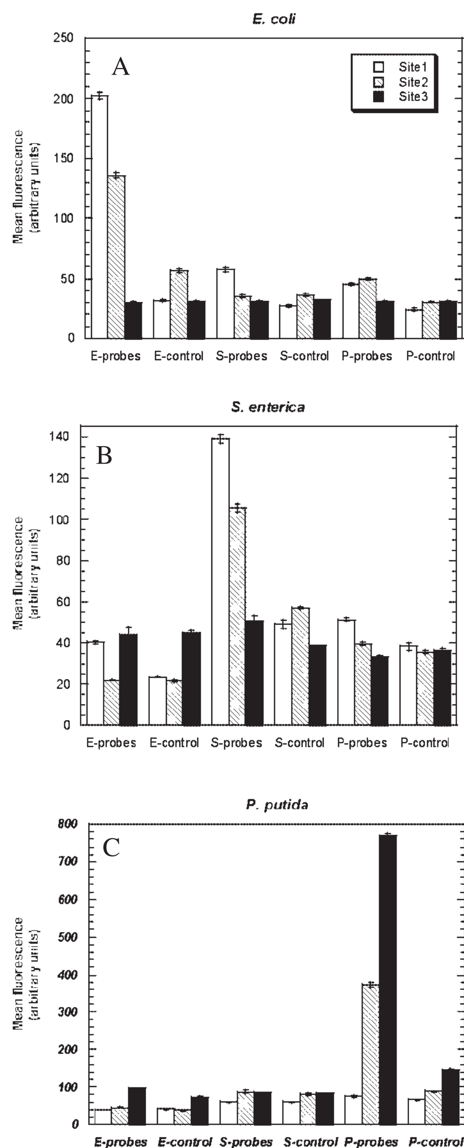

Figure 6. Flow cytometric analysis of bacteria treated with target sites 1–3 probes. Bacteria were incubated at 37°C with 200 nM dabsyl probe, 3 μM helper probes and 2 μM phosphorothioate probe (in cases indicated) for 2 h, then analyzed by using flow cytometry. Mean fluorescence values are from three experiments. E-Probes, *E.coli*-specific dabsyl probe and phosphorothioate; E-Control, *E.coli*-specific dabsyl probe only; S-Probes, *S.enterica*-specific dabsyl probe and phosphorothioate; S-Control, *S.enterica*-specific dabsyl probe only; P-probes, *P.putida*-specific dabsyl probe and phosphorothioate; P-control; *P-putida*-specific dabsyl probe only. (**A**) *E.coli*, (**B**) *S.enterica* and (**C**) *P.putida*.

from cells (18,36,37), which although accurate in allele discrimination, are time consuming and labor intensive.

In this work we focused on the use of QUAL probes to generate a fluorescence change, allowing the discrimination of live bacteria that have highly conserved 16S rRNA sequences. The current method is appealingly simple, requiring only the single step of incubating probes with cells. In the longer term we are interested in applying this method to clinically relevant pathogens; in the present investigation we selected related non-pathogenic strains as an initial test. The *Salmonella* strain we used, an *S.enterica*, is a non-pathogenic relative of the substantially more dangerous *Salmonella typhii* and *S.choleraesuis* (38). *Pseudomonas aeruginosa* is a known pathogen that is particularly dangerous to cystic fibrosis patients, and we selected a non-pathogenic relative, a *P.putida* strain (39,40). Future studies will be needed to evaluate whether the QUAL probes can perform as well with the pathogenic strains.

These are the first data showing genetic discrimination between living *E.coli* and *S.enterica*, which are extremely similar in size and shape and cannot be distinguished by their morphology (41). We targeted a site with a C/A polymorphism, one of the most difficult SNPs to sense, since G–T mismatches lower hybridization T_m only slightly. It was not surprising that brighter signal was obtained with 2′-OMe RNA probes, as 2′-OMe RNA is known to bind RNA targets with greater affinity than DNA (28). Previous studies comparing DNA and 2′-OMe RNA molecular beacons (42) or linear hybridization probes (34) in human cells showed that the 2′-OMe RNA binds tighter but less specifically than the corresponding DNA probes, and our results, showing less sequence discrimination with the modified probes, were consistent with this.

Our data reveal a strong influence of probe length and hybridization temperature on single nucleotide selectivity. No signal was observed for 6mer or 7mer dabsyl probes, while temperature-dependent signals were observed for 8mer and 9mer dabsyl probes. These data suggested a strong dependence on T_m, both for overall signal intensity and for mismatch sensitivity. At 25°C, the *E.coli*-specific probes distinguished the two bacteria, but virtually no selectivity was observed for the *S.enterica*-specific probes (Figure 4A and B), which form a G–T mismatch when bound to *E.coli*. However, when the hybridization temperature was raised to 37°C (Figure 4C and D), substantial discrimination was achieved. Although the flow cytometry results only showed ~1.5-fold increase in fluorescence for the matched probes versus mismatched probes, the bacteria were easily distinguished under the microscope (Figure 5). Again, more non-specific signal was observed in the case of the G–T mismatch (*E.coli* treated with *S.enterica*-specific probes) than in the case of the C–A mismatch (*S.enterica* treated with *E.coli*-specific probes), as expected based on the empirically determined T_m differences of these mismatches.

Our data with optimized-length DNA probes showed that the use of three target sites allowed us to distinguish *E.coli*, *S.enterica* and *P.putida* (Figure 2). Ec/Se584 probes target a moderately accessible region in *E.coli* 16S rRNA (6), and when helper probes were omitted, considerably less signal was observed (data not shown). Target sites 1 and 2 span large accessible regions in *E.coli* 16S rRNA, while target site 3 is in a moderately accessible site spanned by sites that are less accessible (6). The accessibility of 16S rRNA sites for hybridization probes in *S.enterica* and *P.putida* have not been reported, though a consensus model for *in situ* accessibility for prokaryotes has been proposed (43). In the consensus model, the accessibility of target site 1 is undetermined, while the accessibility of target sites 2 and 3 are very similar to the empirically determined accessibilities in *E.coli*. For all three target sites, bright fluorescence was only observed when fully matching sequenced probes were used along with helper probes. However, no signal at all was observed for *P.putida* treated with target site 1 probes, and

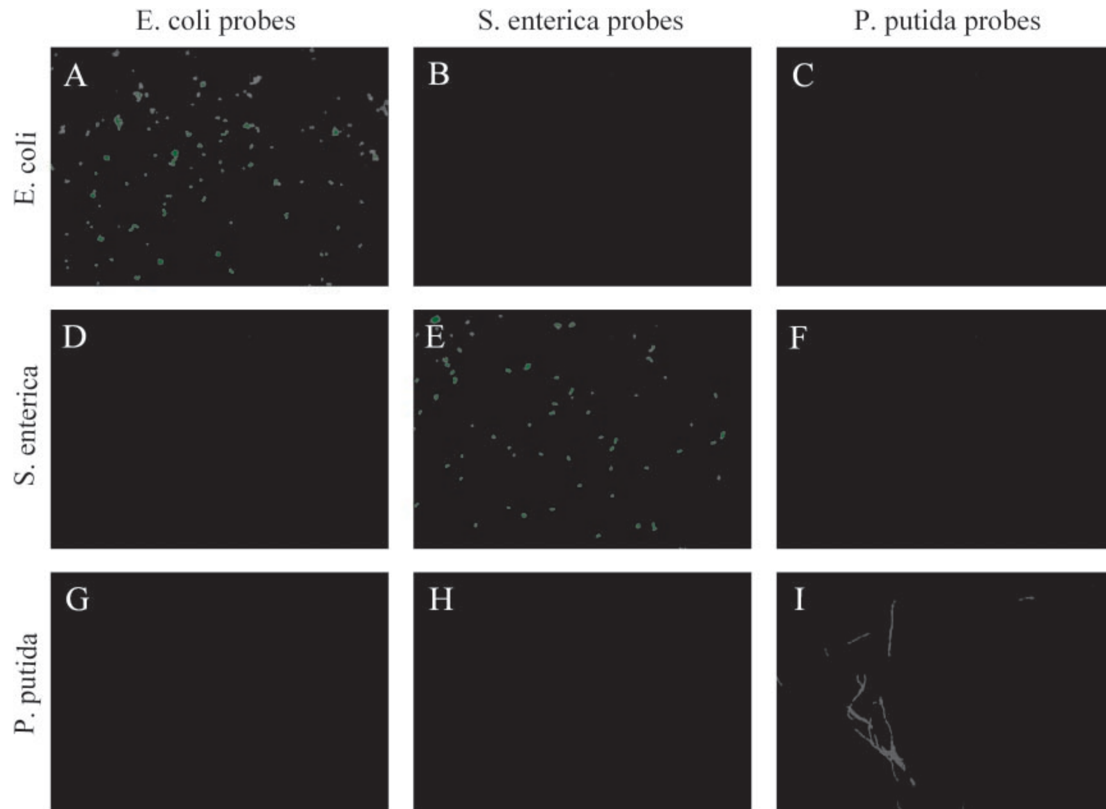

Figure 7. Fluorescence images of *E.coli*, *S.enterica* and *P.putida* treated with target site 2 probes. Bacteria were incubated at 37°C with 200 nM dabsyl probe, 3 μM helper probes and 2 μM phosphorothioate probe (in cases indicated) for 2 h and imaged without any washing steps. (**A–C**) *E.coli*; (**D–F**) *S.enterica*; (**G–I**) *P.putida*. (A, D and G) Bacteria treated with *E.coli*-specific probes are shown; (B, E and H) Bacteria treated with *S.enterica*-specific probes are shown; and (C, F and I) bacteria treated with *P.putida*-specific probes are shown.

also for *E.coli* and *S.enterica* treated with target site 3 probes. This result, however, is largely consistent with Amann's *in situ* accessibility models.

Target site accessibility will remain a factor for experimentation when designing probes for other bacterial strains. However, the present data show that QUAL probes can distinguish bacteria with highly conserved rRNA sequences. Future studies will be aimed at detecting antibiotic-resistant bacteria, which typically have SNPs in their rRNA (44,45), as well as specific identification of pathogenic microorganisms in clinical samples.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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