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Imaging of RNA in Bacteria with Self-Ligating Quenched Probes

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In recent years the field of microbiology has been greatly impacted by the application of fluorescent-labeled oligonucleotides, which have made it possible to identify strains of bacteria and other single-celled microorganisms in a few hours.[1] Standard labeled oligonucleotides, commonly 15−30 nt long, can be hybridized to ribosomal RNAs in fixed bacterial cells, allowing identification by fluorescence microscopy. A large database of rRNA sequences from many microorganisms now exists,[2] allowing the identification of widely different organisms by in situ hybridization.

However, such standard oligonucleotide probes have a number of limitations; for example, they show low selectivity and are generally unable to distinguish related sequences of RNA unless there are multiple nucleotide differences.[1b,3,4] Second, standard probes require careful handling to avoid nonspecific signals. Typically, cells are first fixed (killed, permeabilized, and cross-linked with formaldehyde). Hybridization is followed by several careful washes to remove unbound probes.[1] This preparation takes time, increases the chances of error, and prevents application in live cells.

Here we report on the application of a new class of synthetic quenched DNA probes, the QUAL probe,[5] that combines a nonenzymatic self-ligation reaction with the loss of quenching (Figure 1). Such probes display high selectivity for even single nucleotide differences, and because quenching is efficient they require no washing away of unbound probes to observe the signal. We demonstrate their use for direct detection of RNA sequences at multiple sites in fixed bacterial cells.

Preliminary experiments established that fluorescein-labeled QUAL probes could be used to detect DNAs in solution at single-nucleotide resolution.[5] However, in cellular applications the targeting of RNA is important because there are usually many more copies of RNA in a cell than DNA, thus allowing for greater sensitivity. Moreover, RNA is often more accessible for probing since it contains single-stranded structure.

The self-ligation approach offers the possible advantages of sequence selectivity, rivaling ligase enzymes,[6] and relatively high efficiency on RNA targets (unlike ligases).[7,8] The requirement for no enzyme or reagents invokes the possibility of application in intact cells. However, QUAL probes have not yet been tested with RNA, nor with targets containing secondary structure. In addition, it has not yet been established whether the complex mixture of proteins and nucleic acids in the cell might interfere with recognition and reaction.

To examine these issues, we constructed probes targeted to ribosomal RNAs in the *Escherichia coli* K12 strain MG1655. The sequences of the 16S RNAs (∼1540 nt in length) are known (see Supporting Information). On the basis of recent mapping data we chose four sites (site **1**: positions 181−215; site **2**: 298−335; site **3**: 320−356; site **4**: 873−910) known to be accessible to varying extents to standard fluorescent DNAs.[9] Four probe pairs were constructed: thioate nucleophile probes **1**−**4** (each 18−20 nt in length) and dabsyl-substituted electrophile probes **1**−**4** matched with these (17−20 nt long) (see Supporting Information). The latter probes were fluorescein-labeled at uracil (as a commercial C5-alkenyl conjugate) within 3−4 nt of the dabsyl end group to encourage efficient quenching (Figure 1).

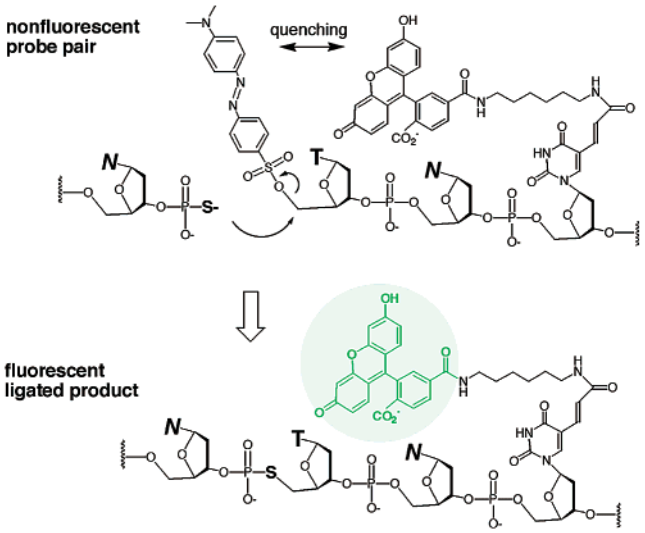

Figure 1. The use of quenched autoligation (QUAL) probe pairs in bacterial RNA sensing. Loss of dabsyl results in "lighting up" of the fluorescent probe, reporting on the bond-forming reaction in real time.

First we tested the probe pair (thioate probe **4** and dabsylate probe **4**) targeted to site **4**. Cells were fixed with paraformaldehyde according to literature methods[10] and were incubated with the probes. Products were imaged by fluorescence microscopy without washing unbound probes from the specimen. After 18 h of incubation we found a distinct green signal from the bacteria (Figure 2a,b). Two controls were also tested. First, the dabsylate probe **4** alone yielded little or no signal (Figure 2c), establishing that both a nucleophile and an electrophile probe are needed to generate the strong signal. Second, pairing thioate probe **4** with electrophile probe **3** also yielded little or no signal (Figure 2d). Both probes are complementary to the ribosomal RNA but at spatially separated sites (553 nt apart), preventing ligation. Thus, adjacent binding of nucleophile and electrophile is necessary for generation of the strong signal. Importantly, the results also establish that noncovalent binding of probes alone is insufficient to generate a signal; this suggests that dabsylate probes are unlikely to give false signals if they adventitiously bind a protein or an unintended nucleic acid target.

A time course at site **4** shows growth in intensity of the signal, reaching a maximum after 6−8 h (Figure 3). For comparison, the

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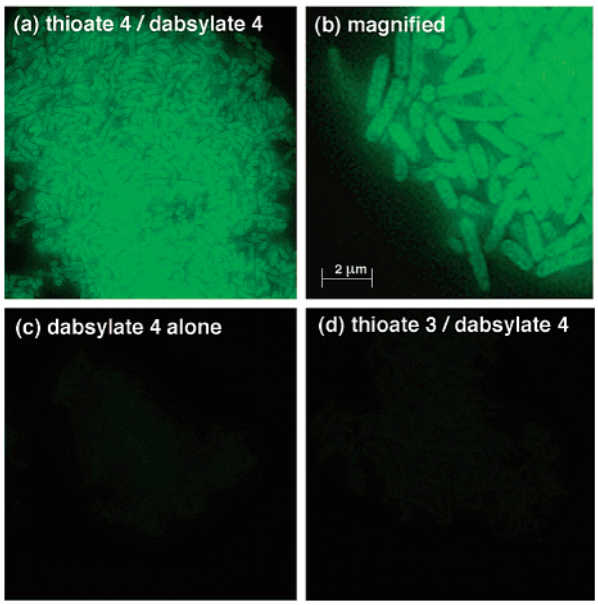

Figure 2. Sensing of site **4** ribosomal RNA in fixed *E. coli* K12 MG1655 cells, showing specific signal with adjacent probe pair and little or no signal with controls. (a,b) Matched thioate **4**/dabsylate **4** probes; (c) dabsylate probe **4** alone; (d) thioate probe **3**/dabsylate probe **4**. Reactions were carried out in a Tris-HCl buffer (pH 7.2) at 37 °C (0.9M NaCl, 0.1% SDS). No washing was done after hybridization.

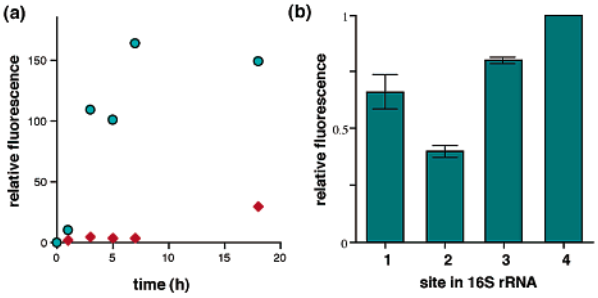

Figure 3. Signal from QUAL probe pairs as a function of time and ribosomal target site. (a) Timecourse for sensing of 16S RNA site **4** with paired thioate probe **4** and dabsylate probe **4** (green circles), or mismatched control using thioate probe **3** and dabsylate probe **4** (red diamonds). (b) Relative amounts of signal in targeting four different sites in 16S RNA. Sequences are given in Supporting Information.

mismatched probe pair shows little signal increase until a small amount of signal is seen at 18 h.

Testing appropriately matched probe pairs at all four 16S rRNA sites revealed a significant signal in all cases (Figure 3b). This establishes that multiple secondary/tertiary structures are targetable by this approach. The amount of signal varies significantly, which establishes that structure influences the outcome, as is true for standard fluorescent DNA probes.[1,9] Site **4**, which we observe to yield the most intense signal of the four with these QUAL probes, is scored as one of the most accessible sites in the 16S rRNA for standard probes as well.[9]

The data show that QUAL probes can be used to detect specific RNA sequences in structured biological targets in cells. This suggests the possibility of their general use in identifying bacterial pathogens by their ribosomal RNAs.

In principle one might use other types of quenched probes, such as beacons[11−13] to detect bacterial rRNAs without washing steps. To date, however, molecular beacons (MBs) have been confronted with difficulties with nonspecific signals. Binding of MBs to any DNA-binding protein in the cell can yield a signal.[14] In a recent cellular study, MBs showed no advantage over standard linear probes, due to low affinity and nonspecific signal.[15] By contrast, QUAL probes are unlikely to yield nonspecific signal even if they do bind a protein, since covalent displacement of the quencher is required. Other mechanisms for releasing the quencher are unlikely: QUAL probes do not react with thiols even at millimolar concentrations.

Future studies will be aimed at development of multicolor QUAL probes for simultaneous sensing of more than one sequence. Also to be studied are applications in identification of pathogenic bacteria, and RNA sensing in living cells.

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Supporting Information Available: Details of probe synthesis, bacterial imaging methods, and complete sequences (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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