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Exploring the in situ accessibility of small subunit ribosomal RNA of members of the domains Bacteria and Eukarya to oligonucleotide probes

Hatice E. Okten[a], L. Safak Yilmaz[b,*], Daniel R. Noguera[c]

[a] Bahcesehir University, Engineering Faculty, Department of Environmental Engineering, 34349 Besiktas, Istanbul, Turkey
[b] Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324, USA
[c] Department of Civil and Environmental Engineering, University of Wisconsin - Madison, 1415 Engineering Drive, Madison, Wisconsin 53706-1691, USA

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ABSTRACT

The principle that the small subunit ribosomal RNA (ssu rRNA) is generally accessible to oligonucleotide probes designed to have high thermodynamic affinity was tested with *Stenotrophomonas maltophilia*, *Rhodobacter sphaeroides*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*. Fluorescein-labeled probes, designed to have $\Delta G_{overall}^{\circ} = -14 \pm 1$ and to avoid the potential of nucleobase-specific quenching, were used to target 20 randomly selected sites in each organism. A site was considered accessible if probe brightness was at least 10 times the background signal. With 30-h hybridizations, 71 out of 80 target sites passed the accessibility criterion. Three additional sites were demonstrated to be accessible with either longer hybridizations, which seemed to have a negative effect on some probes, or the addition of formamide to the hybridization buffer. The remaining 6 sites were demonstrated to be accessible by changing the fluorophore to Cy5, slightly modifying probe lengths, using dual-labeled fluorescein probes, or a combination of these approaches. Probe elongations were only needed in 4 probes, indicating a 95% success in correctly predicting $\Delta G_{overall}^{\circ}$, the key parameter for the design of high affinity probes. In addition, 94% of the fluorescein labeled probes yielded bright signals, demonstrating that nucleobase-specific quenching of fluorescein is an important factor affecting probe brightness that can be predicted during probe design. Overall, the results support the principle that with a rational design of probes, it is possible to make most target sites in the ssu rRNA accessible.

Introduction

Fluorescence in situ hybridization (FISH) is a rapid cultivation independent method for detecting and quantifying specific microorganisms within mixed microbial communities [1–3,24]. In most FISH applications, ribosomal RNA (rRNA) molecules are preferred targets because of their abundance inside cells. In particular, the small subunit rRNA (ssu rRNA) is widely used as a phylogenetic marker [13]. The ssu rRNA sequence has conserved and variable regions [27], a characteristic that is used for defining target sites with the desired level of specificity, which could be from species to domain level [3].

Central to the development of useful FISH applications is the ability to produce hybridizations that maximize sensitivity (i.e., high signal-to-noise ratio) while maintaining specificity towards the targeted organisms. Sensitivity was initially thought to be a function of the location of the target site on the ssu rRNA molecule [4,14] because of possible interference from higher order structures in the ribosome. Following this concept, Fuchs et al. [15] carried out a systematic study to evaluate the effect of target site location by performing hybridizations over the entire 16S rRNA molecule of *Escherichia coli*. Their results were interpreted as demonstrating the existence of inherently inaccessible regions on the 16S rRNA of *E. coli*. On a follow-up publication, Behrens et al. [9] used a similar approach to generate accessibility maps for three additional microorganisms, with the purpose of evaluating whether the location of inaccessible regions found in *E. coli* could be extended to other organisms. They produced a consensus map from the combination of the accessibilities observed with the different organisms. Another publication from the same group of researchers investigated the effect of RNA-RNA and RNA-protein interactions on hybridization efficiency [7]. However a correlation between fluorescence signal intensity and ribosomal structure could not be found.

Searching for alternative mechanisms to understand rRNA accessibility to DNA probes, Yilmaz and Noguera [28] demonstrated that the thermodynamic affinity of the probe to the target site, measured as the overall free energy ($\Delta G_{overall}^{\circ}$) of a reaction including probe-target hybridization as well as probe and target self folding,

* Corresponding author. Tel.: +1 508 856 2636; fax: +1 508 856 6231.
E-mail addresses: lutfu.yilmaz@umassmed.edu (L.S. Yilmaz), noguera@engr.wisc.edu (D.R. Noguera).

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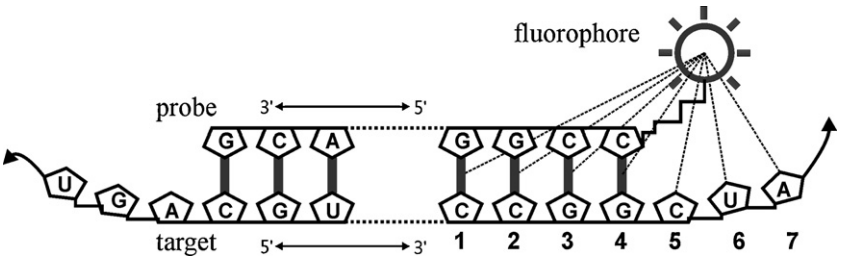

Fig. 1. Illustration of quenching neighborhood where rRNA nucleotides play a role in nucleobase-specific quenching. Key positions are numbered in accordance with Table 1.

was a good predictor of probe brightness. In addition, it was suggested that the absence of formamide in the hybridization buffer could result in kinetic limitations when short hybridization periods were used. Applying these concepts, Yilmaz et al. [30] used thermodynamic principles for probe design and extended hybridization periods to demonstrate that the entire 16S rRNA of *E. coli* could be made accessible to DNA probes, and thus, that there were no intrinsically inaccessible sites on the ssu rRNA of this organism. Yilmaz et al. [30] concluded that the most important criterion for efficient hybridization was the establishment of DNA/RNA hybrids with an overall thermodynamic affinity of −13 kcal/mol or lower. In addition, they also showed that even with high hybridization efficiency, signal intensity of fluorescein-labeled probes might have been low due to fluorophore quenching, which is known to depend on the close proximity of specific nucleotides [12,23].

The thermodynamic affinity approach for the design of FISH probes has so far been proven to be effective for *E. coli* [28,30], but an experimental demonstration that the concept can be generalized to other organisms has not been provided. Therefore, the objective of this study was to determine whether random target sites in the ssu rRNA of other organisms could also be made accessible to DNA probes when thermodynamic and kinetic limitations were overcome. To achieve this goal, FISH experiments with four organisms representing different phylogenetic groups were conducted using flow cytometry methods described in earlier publications [29,30]. In order to minimize the problem of probe quenching in this analysis, we also developed an empirical formulation to predict nucleobase-specific fluorescein quenching.

Materials and methods

Microbial growth conditions

Stenotrophomonas maltophilia was grown aerobically on Lennox LB broth (Fisher Biotech, Fair Lawn, NJ) at 25 °C. *Rhodobacter sphaeroides* was grown either aerobically or photoheterotrophically at 30 °C, with Sistrom's medium [20] containing succinate as the organic substrate. *Bacillus subtilis* was grown aerobically on LB broth at 37 °C. *Saccharomyces cerevisiae* was grown at 37 °C on a medium that contained 20% Bacto™ Yeast Extract (Becton and Dickinson and Company, Sparks, MD), 40% Bacto™ peptone (Becton and Dickinson and Company, Sparks, MD) and 40% D(+)-glucose (Acros, NJ). Growth curves were generated for each organism by measuring optical density at 600 nm.

For FISH experiments, cells were harvested in the exponential growth phase. Optical density values at fixation for *S. maltophilia*, *R. sphaeroides*, *B. subtilis*, and *Sc. cerevisiae* were 0.40 ± 0.05, 0.70 ± 0.15, 0.85 ± 0.05, and 1.5 ± 0.1, respectively. *S. maltophilia*, *R. sphaeroides* and *B. subtilis* cells were fixed with 4% paraformaldehyde for 30 min at room temperature. *Sc. cerevisiae* was also fixed with 4% paraformaldehyde and at room temperature, but the fixation period was 3 h, following the procedures of Amann et al. [6]. Longer fixation periods may potentially inactivate RNases that otherwise may degrade rRNA during hybridization. Fixed cells were pelleted, resuspended in 1× PBS (phosphate buffered saline), and stored at 4 °C until hybridization. Paraformaldehyde fixation worked well with log-phase *B. subtilis* in this study, but in general, paraformaldehyde fixation is not recommended for Gram positive organisms, as its effectiveness is growth phase dependent.

Probe synthesis

Mono- and dual-labeled probes were used in this study. All mono-labeled probes were synthesized at the University of Wisconsin Biotechnology Center with fluorescein phosphoramidite (FAM) (applicable to all probes) or Cy5-CE phosphoramidite (applicable only to probes suspected of experiencing nucleobase-specific quenching) attached to the 5′ end of the probe. Dual-labeled probes with FAM attached to both 5′ and 3′ ends were purchased from Sigma–Aldrich (Saint Louis, MO).

Evaluation of fluorophore quenching

The co-variance between probe brightness and the identity of nearby rRNA nucleotides to the 3′ end of target site (Fig. 1) was evaluated with multi-way analysis of variance (ANOVAN), using the "anovan" function of MATLAB (The MathWorks Inc., Natick, MA). Statistical *p*-value output by this function characterized the relevance of nucleotide position and type to quenching. The "solver" routine of MS Excel (Microsoft Corp., Redmond, WA) was employed for obtaining the scores yielding maximum correlation (Table 1) and the corresponding best fitting line that described the relationship between probe brightness and the total probe quenching factor (Fig. 2).

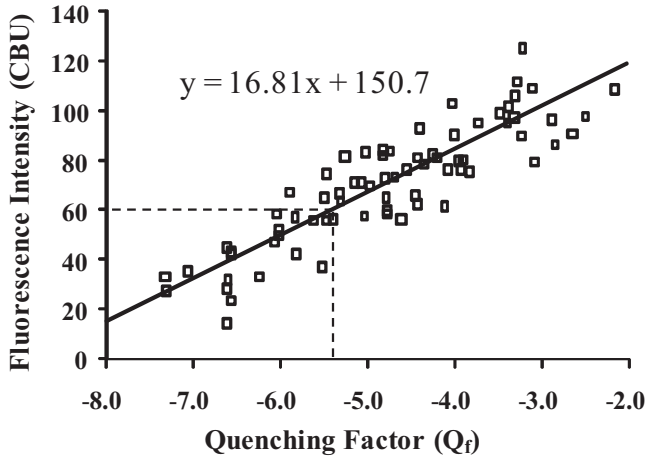

Fig. 2. Maximized correlation between quenching factor and fluorescence intensity for high affinity probes from Yilmaz et al. [30], upon 96 h hybridization periods. Solid curve is the best fitting line. Dashed line indicates the threshold used for probe selection in this study.