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Label-free detection of 16S ribosomal RNA hybridization on reusable DNA arrays using surface plasmon resonance imaging

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Summary

In this paper, we describe the detection of bacterial cell-extracted 16S ribosomal RNA (rRNA) using an emerging technology, surface plasmon resonance (SPR) imaging of DNA arrays. Surface plasmon resonance enables detection of molecular interactions on surfaces in response to changes in the index of refraction, therefore eliminating the need for a fluorescent or radioactive label. A variation of the more common SPR techniques, SPR imaging enables detection from multiple probes in a reusable array format. The arrays developed here contain DNA probes (15–21 bases) designed to be complementary to 16S rRNA gene sequences of *Escherichia coli* and *Bacillus subtilis* as well as to a highly conserved sequence found in rRNAs from most members of the domain Bacteria. We report species-specific hybridization of cell-extracted total RNA and *in vitro* transcribed 16S rRNA to oligonucleotide probes on SPR arrays. We tested multiple probe sequences for each species, and found that success or failure of hybridization was dependent upon probe position in the 16S rRNA molecule. It was also determined that one of the probes intended to bind 16S rRNA also bound an unknown protein. The amount of binding to these probes was quantified with SPR imaging. A detection limit of 2 μg ml^{-1} was determined for fragmented *E. coli* total cellular RNA under the experimental conditions used. These results indicate the feasibility of using SPR imaging for 16S rRNA identification and encourage further development of this method for direct detection of other RNA molecules.

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Introduction

Molecular phylogenetic analyses based upon 16S rRNA sequence heterogeneity has revealed an astounding diversity of microbial life in natural environments, much of which is recalcitrant to standard cultivation techniques (Bintrim *et al*., 1997; Hugenholtz *et al*., 1998). To understand better the contribution of these uncultured microorganisms to ecosystem processes, we are developing the molecular tools necessary to access functions encoded by environmental microorganisms (Rondon *et al*., 2000) and to detect alterations in their abundance and metabolic activities in response to environmental factors (Rondon *et al*., 1999; Simon *et al*., 2000).

One essential tool for molecular analysis of complex microbial assemblages is the ability to query large nucleic acid datasets, such as rRNA sequences. DNA array technology has the potential to provide quantitative information on complex mixtures in a fraction of the time required by traditional methods (Fodor, 1997). However, application of DNA arrays to the quantitative measurement of RNA is limited by single-use array formats and the laborious techniques and high costs required for fluorescent labelling and detection (Lockhart *et al*., 1996).

Surface plasmon resonance (SPR) is a label-free technique now widely used to monitor affinity interactions between molecules of biological interest by measuring changes in refractive indices (Fivash *et al*., 1998; Frutos and Corn, 1998). Within the last decade, SPR has been used to measure the label-free binding of biological molecules onto chemically and biologically modified surfaces (Silin and Plant, 1997). A major drawback of the instrumentation used for conventional SPR is its inability to detect large numbers of molecular interactions simultaneously.

A variation of this technique, SPR imaging (Rothenhausler and Knoll, 1988; Hickel *et al*., 1989), allows simultaneous analysis of multiple molecular probes for affinity against a target molecule or mixtures of target

molecules (Brockman *et al*., 2000). Surface plasmon resonance imaging has recently been shown to be an effective tool for the analysis of DNA hybridization (Jordan *et al*., 1997; Thiel *et al*., 1997), detection of RNA oligonucleotide hybridization (Nelson *et al*., 2001), and DNA–protein interactions (Brockman *et al*., 1999; 2000; Frutos *et al*., 2000).

Our SPR imaging device employs light from a collimated white light source that illuminates a glass prism at a fixed angle (Fig. 1). A glass slide coated with a thin gold film carrying a patterned array of DNA probes is optically coupled to the prism. Light at 800 nm interacts with the patterned gold film from behind, creating surface plasmons. Reflectivity of the incident light is attenuated upon the creation of the surface plasmons, and the amount of attenuation is determined by the index of refraction closer than 500 nm from the gold film. Adsorption of molecules such as nucleic acids onto the surface affects the index of refraction, thereby causing a change in the reflectivity of incident light. The resulting changes in reflectivity are monitored with a CCD camera. Recent improvements in the design of the SPR imaging system have yielded improved image contrast and sufficient sensitivity to clearly detect interactions between biological molecules without amplification (Nelson *et al*., 1999).

Using UV-photopatterning techniques (Tarlov *et al*., 1993), we employ a multistep procedure for creating DNA arrays on gold surfaces for use with SPR imaging (Brockman *et al*., 1999). Areas on the gold surface intended for spotting with DNA probes are surrounded by regions modified with a removable hydrophobic protecting group that confines each DNA probe to its respective array position on the surface. The 5′ thiol-modified DNA probes are attached covalently with a bifunctional linker that is bound to an amine-terminated monolayer on the gold surface. After the DNA has been spotted, the protecting group is removed and replaced with methoxypoly(ethylene glycol) propionic acid (PEG) groups, which resist nonspecific adsorption by a variety of target analytes. A depiction of the final array format on the gold surface is shown in Fig. 1.

We chose 16S rRNA as the first RNA molecule for analysis by SPR imaging owing to its well-characterized structure, its relative abundance in metabolically active cells, and its utility in microbial identification (Dennis and Bremer, 1974; Maidak *et al*., 2001). The expanding database of 16S rRNA gene sequences available at the Ribosomal Database Project (http://rdp.cme.msu.edu) allows investigators to relate potential rRNA probe sequences to phylogenetic classifications, enabling the development of arrays for phylogenetic characterization of complex microbial communities (Woese, 1987; Head *et al*., 1998; Hugenholtz *et al*., 1998). A principle challenge to using oligonucleotide probes to detect 16S rRNA molecules is its high degree of secondary structure, which can inhibit binding to these probes (DeLong *et al*., 1989; Frischer *et al*., 1996). Targeting a specific rRNA molecule with multiple oligonucleotides has proven effective in identifying probes that can successfully hybridize; in addition, each successful probe may give different levels of phylogenetic resolution. For example, others have used multiple fluorophore-conjugated oligonucleotide probes directed to both evolutionarily conserved and taxon-specific 16S rRNA regions to simultaneously reveal the members of the domain Bacteria as well as specific bacterial phyla in an environmental sample (Stahl *et al*., 1989; Whiteley and Bailey, 2000). Guschin *et al.* (1997) demonstrated the use of DNA arrays for the detection of rRNA and rDNA, using

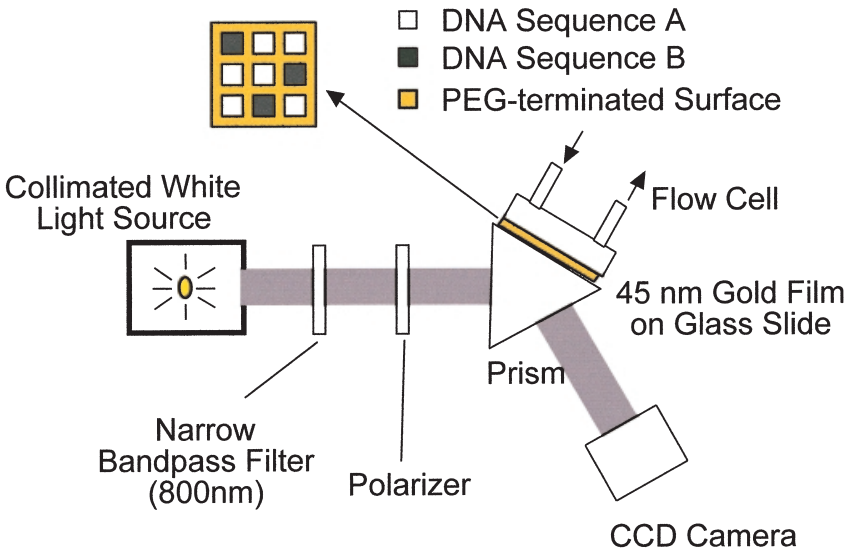

Fig. 1. Schematic of SPR imager instrument and surface geometry used in these experiments. Collimated white light passes first through a narrow bandpass filter (800 nm), a polarizer and a glass prism. A glass slide with a thin (45 nm) coating of gold is optically coupled to the prism. The surface of the gold film is chemically modified as depicted in the figure. Methoxypoly(ethylene glycol) propionic acid (PEG) groups surround the DNA spots and prevent non-specific adsorption onto the background of the array. Light at 800 nm interacts with the patterned DNA array on gold from behind, creating surface plasmons. The array is imaged in the presence of hybridization buffer; an RNA target sample is introduced using the flow cell. Change in the index of refraction at DNA spots where hybridization adsorption of RNA occurs affects the per cent reflectivity of the incident light. Changes in per cent reflectivity are detected with a CCD camera.

polyacrylamide gel pads to immobilize phyla-specific probes. This approach enabled the simultaneous detection of a variety of microorganisms in a single experiment.

Our approach offers the advantage of label-free detection of rRNA hybridization to a probe array, as well as a reusable format, which may allow for analysis of multiple samples using a single array. This is in contrast to many of the current methods for use of DNA arrays, which rely upon reverse transcription of RNA, fluorescent labelling of RNA samples for detection, and/or single-use arrays. In addition, the use of arrays allows us to target a single organism with multiple probes, as well as screen for multiple organisms in a sample. We previously reported the use of reusable DNA arrays for the label-free detection of RNA oligonucleotides at concentrations as low as 10 nm using SPR imaging (Nelson *et al*., 2001). Whereas demonstrating the ability to detect RNA with SPR imaging, this previous study did not address issues related to the hybridization of cell-extracted RNA onto a DNA array. In this paper, we extend our results to hybridization of rRNAs in total cellular RNA preparations; we also report first steps toward the use of SPR imaging to detect differential hybridization to taxon-specific probes.

Results and discussion

DNA probe design and RNA secondary structure

Because 16S rRNA is replete with secondary structure (Lockhart *et al*., 1996), careful consideration in DNA probe design and pretreatment of target RNA was necessary to achieve the desired hybridization. We tested 15 DNA probe sequences for hybridization to RNA isolated using various methods; those that were found to bind 16S RNA (as well as a negative control) are listed in Table 1. Other probe sequences tested that did not hybridize successfully to 16S rRNA are available from the authors (http://http://www.plantpath.wisc.edu/goodmanlab/addinfo/SPR.html). In some cases regions of the 16S rRNA molecule deemed accessible to oligonucleotide probes in whole cell hybridizations (Fuchs *et al*., 1998) were used as a general guide for probe selection, but our results also showed that each probe must be independently verified for hybridization to its respective rRNA molecule on an SPR array. For example, we found in preliminary experiments that EUB338, a universal prokaryotic probe used for whole cell hybridization *in situ* (Amann *et al*., 1990), failed to bind 16S rRNA from any species tested when used as a probe on our DNA arrays. Inspection of the EUB338 sequence revealed a predicted self-complementary sequence forming a hairpin structure that could be avoided by shifting the sequence used by four nucleotides; the newly designed probe (EUB342; Table 1) was found to bind successfully to RNA of all three microbial species (*E. coli, B. subtilis* and *Bacillus cereus*) used as sources of rRNA in our experiments.

We found two methods of RNA denaturation, heat treatment and chemical fragmentation, that resulted in hybridization of 16S rRNA to the DNA array. Without either prior fragmentation or boiling of cellular RNA, no hybridization signal was detected. Heat treatment consisted of boiling the RNA sample for five minutes, followed by immediate immersion in an ice bath. Gel electrophoresis showed that this method did not result in fragmentation of the 16S rRNA, giving a band of the same size as non-heat treated rRNA (data not shown).

Detection of rRNAs from total cellular RNA preparations

For the results shown in this paper, we isolated total RNA from bacterial cultures in mid-log phase growth and chemically fragmented the RNA samples for analysis with SPR imaging. Figure 2A shows results obtained with SPR imaging after sequential hybridization of *E. coli* and *B. subtilis* RNAs to a DNA array for one hour. The DNA array

Table 1. Oligonucleotide sequences for probe (surface immobilized) DNA. Sequences of probes with symbols beginning with an 'E' or 'B' are complementary to the 16S rRNAs of *E. coli* or *B. subtilis* respectively. A 'universal' bacterial probe, EUB342, was designed to bind to the 16S rRNAs of most bacteria.

Symbol	Base position[a]	Sequence	Phylogenetic specificity[b]
E186	186–205	5′-GTCCCCCTCTTTGGTCTTGC-3′	Enteric bacteria and relatives
E434	434–449	5′-CTCCCCGCTGAAAGTA-3′	Enteric bacteria and relatives
E491	491–505	5′-CGGTGCTTCTTCTGC-3′	*Proteobacteria*, other divisions
B186	186–206	5′-CTTTTATGTTTGAACCATGCG-3′	*B. subtilis* subgroup
B434	434–449	5′-TTCCCTAACAACAGAG-3′	*Bacillus-Streptococcus* subdivision
B491	491–506	5′-CGTGGCTTTCTGGTTA-3′	*Bacillus-Streptococcus* subdivision
EUB342	342–357	5′-ACTGCTGCCTCCCGTAG-3′	'Universal' prokaryotic[c]
NC	Negative control	5′-GGATGTGTGTGGAGTGTTAGAAAG-3′	None

a. Positions are relative to the published *E. coli* 16S rRNA sequence (Blattner *et al*., 1997).
b. Based upon the database of rRNA sequences available at the Ribosomal Database Project.
c. EUB342 does not have 100% homology to all of the rRNA sequences within the RDP database, but is predicted to bind to the majority of the rRNA sequences in this database.

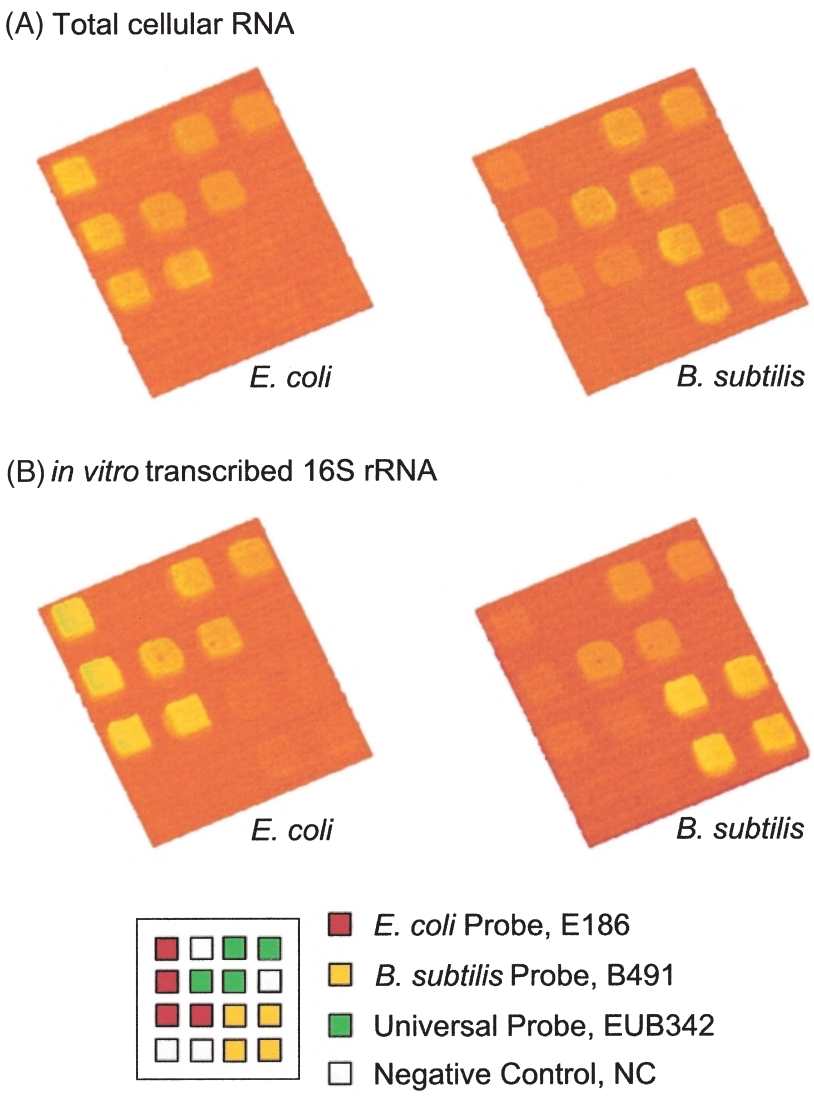

Fig. 2. SPR images showing representative hybridization of total RNA isolated from a cell culture (A) or *in vitro* transcribed rRNA (B) onto DNA arrays. Hybridization onto the array is indicated by a change in the percent reflectivity of incident light. The pattern used for immobilization of single stranded DNA probes is shown in the legend.
A. A 35 μg sample of total RNA from *E. coli* was first exposed to the array for 1 h (left). After measurement, the array was denatured using 8 M urea. The experiment was repeated with the same amount of total RNA from *B. subtilis* (right).
B. A 50 μg sample of *in vitro* transcribed 16S ribosomal RNA from *E. coli* was first exposed to the array for 1 h (left). After denaturation, the experiment was repeated with the same amount of *in vitro* transcribed 16S rRNA from *B. subtilis*. The array used was hybridized and denatured more than 20 times before the data shown were taken. A 4×4 section of the same 10×12 array is shown for all images in the figure.

contained probes for both species, EUB342 (our 'universal' prokaryotic probe), and a negative control, NC. In the left panel of Fig. 2A, a 35 μg sample of fragmented total RNA from *E. coli* was first exposed to the array. Upon introduction of the sample, the most significant change in percent reflectivity (3.9%) occurred at array features containing the *E. coli* probe, E186. This corresponds to a per cent reflectivity change of 0.11% μg^{-1}. For array features with the universal probe, EUB342, the change in percent reflectivity was 0.075% μg^{-1}. A very small amount of adsorption was recorded onto array features containing other sequences, including a 0.005% μg^{-1} change in reflectivity at features with the *B. subtilis* probe, B491, and a nearly undetectable amount (0.0005% μg^{-1}) at features that contained the negative control probe (NC). Even less adsorption to the PEG background was observed, showing the effectiveness of our surface chemistry in eliminating background adsorption. After exposure to the *E. coli* sample, the array was denatured using 8 M urea. An SPR image taken after denaturing showed no detectable difference in the state of the array compared to that before hybridization. The hybridization experiment was repeated with the same amount of fragmented total cellular RNA from *B. subtilis* for 1 h (Fig. 2A, right panel). Introduction of the sample isolated from B. *subtilis* produced the strongest change in per cent reflectivity at features containing the universal probe, EUB342, and the *B. subtilis* probe, B491, with changes in per cent reflectivity of 0.099% μg^{-1} and 0.097% μg^{-1} respectively. Results with *B. subtilis* rRNA again showed virtually undetectable adsorption to the negative control (0.009% μg^{-1}) and to the PEG background, but did show significant signal (0.044% μg^{-1}) at features containing the *E. coli* probe, E186. The cross-hybridization of *B. subtilis* total RNA to the *E. coli*-specific E186 probe was consistent in repeated experiments and is addressed in the next section.

The highly reusable nature of the surface chemistry is evidenced by the fact that the array was hybridized and denatured more than 20 times before the data shown in Fig. 2A and B were collected. Hybridization of perfectly matched oligonucleotides to the same probes on this array at 100 nM showed less than 15% variability in signal for multiple hybridizations/denaturing steps on the same array (data not shown). In a previous paper, we demonstrated we could hybridize and denature RNA oligonucleotides to a surface more than 20 times with only a 5% drop in signal (using 150 nm RNA target concentration, which is equivalent to 80% surface saturation) using the same procedures described here (Nelson *et al*., 2001).

An important consideration for potential users of SPR imaging is the detection sensitivity, which is, without signal amplification, generally lower than that achievable with fluorescence-based methods. We previously reported the detection limit for SPR imaging to be 10 nM for unlabelled DNA and RNA oligonucleotides (18-mers) using DNA arrays (Nelson *et al*., 2001). Amplification can be used to enhance the SPR signal for more sensitive detection of nucleic acids. For example, gold nanoparticle-tagged DNA has been used as a detection step in a 'sandwich assay' to amplify the SPR signal from DNA adsorption onto a DNA array, resulting in a detection limit of 10 pM for 24-mer oligonucleotides (He *et al*., 2000). In our experiments, the lower limit of detection for fragmented total *E. coli* RNA was determined by successive dilution. We found our lower limit of detection for fragmented RNA sample to be 2 μg ml^{-1} (data not shown). No signal was quantified above noise at 1 μg ml^{-1} for fragmented RNA. Total RNA that was boiled rather than fragmented before introduction into the SPR flow cell resulted in a slightly higher signal (data not shown) compared to that from fragmented RNA. This result is probably because of the fact that SPR response is proportional to molecular weight, i.e. larger molecules adsorbed to the surface result in greater signal intensity.

Characterization of non-specific binding

In order to characterize the nature of the non-specific binding of a component of the *B. subtilis* RNA samples to the E186 probe, we conducted several experiments, including the use of different methods for the isolation or generation of rRNA. We first examined whether the non-specific binding could be reduced by increasing hybridization stringency. The methods used to increase stringency were: (i) increasing the hybridization temperature; (ii) decreasing NaCl concentration; and (iii) adding urea to the hybridization buffer. Use of formamide to increase stringency was also attempted, but it was found to degrade both the PEG background and the immobilized DNA (other surface chemistries may not be similarly affected). The results of these tests showed that while increased stringency led to the complete absence of hybridization to the B491 probe from the *B. subtilis* RNA sample, none of these steps reduced the non-specific binding to the E186 probe. This result suggested that the binding to the E186 probe was not caused by RNA. We next incubated the *B. subtilis* RNA sample with RNAse A for 30 min at 37°C to the point where there was no observable RNA by gel electrophoresis. However, SPR imaging of the remaining reaction product showed that the SPR imaging signal at the E186 probe remained. We then tested whether the SPR signal changed upon treatment of samples with proteinase K. The results showed that the signal was sensitive to proteinase treatment, but only if proteinase K treatment was preceded by RNAse treatment. These results suggest the existence of a ribonucleoprotein complex(es) in *B. subtilis* RNA preparations that are resistant to proteolytic degradation and adsorb to the E186 probe. This adsorption would also occur in experiments that use fluorescence-based detection methods, and could potentially interfere with RNA hybridization. In such an experiment, an interaction such as this would likely have gone undetected, underscoring the unique properties and challenges of SPR-based detection.

We also compared the results obtained with total cellular RNA with other methods for isolating or generating rRNA using SPR imaging. We tested hybridization to the same array used for total cellular RNAs with *in vitro* transcribed 16S rRNA from the same organisms. The results of this experiment are shown in Fig. 2B. Compared to the total RNA preparations, *in vitro* transcribed rRNA contains only one RNA species and lacks ribosomal proteins and other cellular components that might contaminate cellular RNA preparations. The experiment was conducted with 50 μg samples of *in vitro* transcribed 16S rRNA for both *E. coli* and *B. subtilis*. Data obtained using *in vitro* transcribed *B. subtilis* rRNA demonstrated significantly less hybridization at probe E186 than was observed using cellular RNA under identical hybridization conditions on the same array (compare Fig. 2A and B). These results are consistent with the hypothesis that protein present in the *B. subtilis* total RNA sample contributed to the binding to the E186 probe (Fig. 2A).

Besides the difference in adsorption to features modified with probe E186, SPR imaging results using *in vitro* transcribed 16S rRNA from either *E. coli* or *B. subtilis* (Fig. 2B, left and right panels respectively) were, in general, similar to those obtained with total cellular RNA. For example, the change in reflectivity upon hybridization of *E. coli in vitro* transcribed rRNA to probe EUB was 3.9%, or 0.078% μg^{-1} RNA, compared to 0.075% μg^{-1} RNA observed for total cellular *E. coli* RNA binding to this probe. The only other difference noted between results from RNA isolated from cell cultures was that *in vitro*

transcribed RNA hybridized to DNA probes without fragmentation or boiling.

We believe the interference with hybridization alleviated by boiling or fragmentation of cellular RNAs is likely caused by the significantly greater secondary structure of RNA isolated from cell cultures. For comparison of signal, *in vitro* transcripts were also fragmented. We observed a ~40% higher signal for a 50 μg sample (100 μg ml^{-1}) of non-denatured *in vitro* transcribed rRNA compared to otherwise identical but fragmented rRNA transcripts (boiled, non-fragmented total cellular RNA showed only slightly higher signal than fragmented total cellular RNA).

In addition, we isolated both *E. coli* and *B. subtilis* intact ribosomes, and observed that the hybridization of these rRNA samples (which include 5S, 16S and 23S rRNAs) was very similar to those observed for total cellular RNA. Despite multiple rounds of phenol extraction after fragmenting the *B. subtilis* rRNA preparations, and the inability to detect any proteins in these preparations by PAGE, we still observed the non-specific adsorption to probe E186 (data not shown). This latter result suggests that the protein(s) binding to the E186 probe is ribosome-associated. These results clearly indicate that sample purity is an important consideration for SPR detection of RNA, in particular the removal of all contaminating proteins. The development of alternative methods for increased sample purity, as well as compatible methods for more stringent hybridization buffer conditions, are currently the focus of development in our laboratories.

Quantification of signal strength for multiple probe hybridizations

Hybridizations observed with SPR imaging can be quantified by integrating the signal strength, and expressing the results in terms of the change in per cent reflectivity per microgram of RNA added to the SPR flow cell. We showed previously that the relative amount of hybridization to a DNA probe remained approximately linear with respect to the integrated SPR imaging signal for changes in per cent reflectivity of up to 10% (Nelson *et al.*, 2001). In Fig. 3, we show the quantitative results from an experiment in which multiple probes were used simultaneously for detection of the 16S rRNAs of *E. coli* and *B. subtilis*. Because each probe will exhibit different hybridization kinetics with respect to its target rRNA, the quantitative results from different probes targeting the same rRNA species are not directly comparable; however, confidence in interpreting the results of an array hybridization as indicating the presence of a particular RNA species is greatly enhanced by the inclusion of multiple probes targeting each taxon and by normalizing the hybridization signal with domain-level probes (Fox *et al.*, 1980; Guschin *et al.*, 1997; Liu *et al.*, 2001). Results for total RNA are shown in Fig. 3A and for

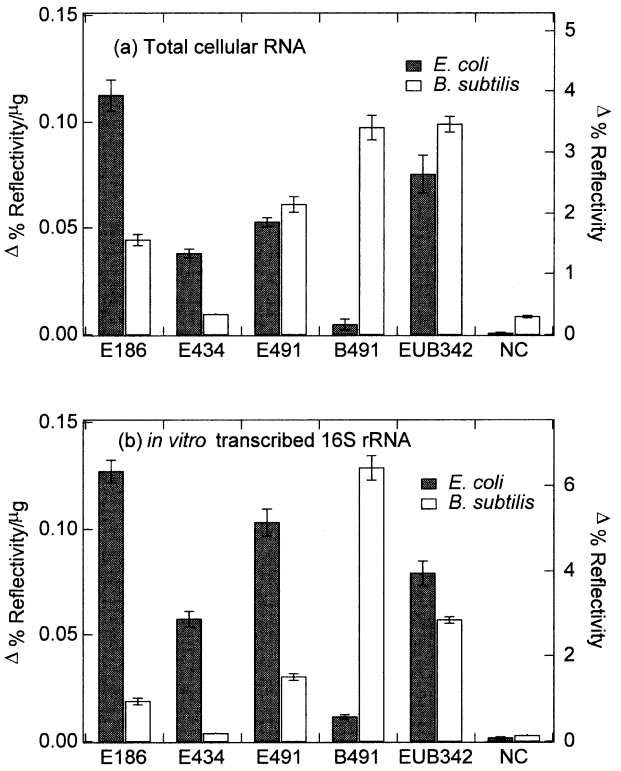

Fig. 3. Quantification of the change in per cent reflectivity due to RNA hybridization for a multiple probe experiment. Both total change in percent reflectivity (right axis) and change in per cent reflectivity per μg of RNA sample (left axis) are shown. Total cellular RNA is shown in A. *In vitro* transcribed rRNA is shown in B. Error bars represent standard deviation for four different probe spots in a given hybridization on the same array. The experiment was conducted twice on two separate arrays.

in vitro transcribed RNA in Fig. 3B. In Fig. 3, the change in per cent reflectivity per μg of RNA is plotted for each probe. Total change in per cent reflectivity for these experiments is close to 5%. Results in Fig. 3A show that for total cellular RNA, probes E186, E434, and B491 clearly distinguish *E. coli* from *B. subtilis* ($P < 0.0001$; one-tailed Student's *t*-test), while probe E491 shows approximately equal binding for both *E. coli* and *B. subtilis* total RNA. In contrast, the hybridization of *in vitro* transcribed rRNAs to probe E491 resulted in significantly greater signal with *E. coli* rRNA ($P < 0.0001$; one-tailed Student's *t*-test). These results demonstrate the variable hybridization of cellular RNAs to different probes, and the ability of SPR imaging to provide quantitative results.

Conclusions

In this paper we have demonstrated detection of cell-extracted 16S rRNAs using SPR imaging. These results indicate that label-free detection of rRNA from bacteria is possible in less than two hours using a standard commercial kit for RNA extraction and allowing one hour for RNA

hybridization. Detection of rRNA hybridization to DNA arrays is a first step toward our long-term goal of developing SPR imaging technology for environmental monitoring of complex microbial assemblages. The level of sensitivity and sample purity required for SPR imaging experiments are still challenges to the general utility of the method. We expect that future work to enhance both sample purity and the stringency of the hybridization reaction in an SPR flow cell will make this technique an attractive alternative to current methods.

Minimization of sample volumes can also contribute to the feasibility of SPR imaging measurements of RNA. Preliminary results of ongoing studies indicate that RNA samples can be individually spotted with as little as 40 nl of target sample. We calculate that at this level, label-free detection of as little as 2 femtomole of RNA will be possible per array feature. When the rRNA detection limit determined here is considered, that translates to detection of 0.1 ng rRNA per feature, or the amount of rRNA from ~5000 metabolically active *E. coli* cells (Neidhardt and Umbarger, 1996). Similarly, recent work in our laboratory has shown that the current SPR reaction chamber can be replaced with microfluidic channels to reduce sample volumes from 500 μl to 1 μl (Lee *et al*., 2001). Additionally, array features sizes can be significantly reduced with widely available arraying equipment, enabling increased information density. Because of inherent limitations, array features must be larger than the propagation length of the surface plasmon (25 μm at 830 nm) (Brockman *et al.,* 2000), however, this feature size limitation will still allow the number of DNA probes that can be arrayed on a single chip to increase from hundreds to a maximum of 40 000 cm^{-2} (based on a 50 μm feature size). With increasing improvements, we expect that SPR imaged microarrays will become a useful tool for microbial ecological studies in natural environments.

Experimental procedures

Reagents

All chemicals were obtained from Sigma Chemical (St Louis, MO), unless otherwise indicated. Oligonucleotides were synthesized by the University of Wisconsin's Biotechnology Facility, and included a 5′ thiol modifier C6 as well as a 15-T spacer prior to the rRNA complementary sequence to provide additional spacing away from the gold surface.

SPR imaging

Details of the SPR imaging apparatus and experiments are available from the authors at http://www.plantpath.wisc.edu/goodmanlab/addinfo/SPR.html as well in previous publications, where instrumental parameters (http://sprimager.com; Nelson *et al*., 1999; 2001), and details of array fabrication, DNA attachment and specific materials used are provided (Brockman *et al*., 1999; Frutos *et al*., 2000). Probe DNAs with a 5′ thiol modifier were covalently attached to gold using the bifunctional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC). All hybridization experiments, unless otherwise indicated, were conducted at 27°C in a buffer of 20 mM phosphate (pH 7.7), 200–300 mM NaCl, and 1 mM EDTA.

DNA probe selection

We selected rRNA regions to target with DNA probes by first aligning *Escherichi coli* and *Bacillus subtilis* rRNA sequences, and identifying the regions of sequence difference. DNA probes were designed based upon the extent of sequence differences between the two respective rRNAs while avoiding sequences with features (e.g. hairpin loops) expected to complicate or interfere with hybridization to the probes. Probes in this study were not designed to distinguish *E. coli* and *B. subtilis* from other closely related species, and each of the probes exhibits varying degrees of phylogenetic resolution (Table 1). We also took advantage of the availability of the complete genome sequences of *E. coli* and *B. subtilis* to avoid probe sequences that might bind to other abundant RNA molecules made by the cells. In order to access the availability of each probe spot for hybridization, perfectly matched DNA oligonucleotides complements were first hybridized to the surface array at a concentration of 100 nM. For all 15 probes tested, the oligonucleotide complements successfully hybridized, demonstrating their accessibility for hybridization to their respective rRNA targets. The inability of some of these probes to hybridize to rRNA is therefore attributed to the fact that they targeted inaccessible regions of the rRNA molecule.

Total cellular RNA preparation

Escherichia coli strain DH10B (Gibco-BRL, Rockville, MD), *B. subtilis* strain 168 (ATCC 23857) and *B. cereus* strain UW85 (Handelsman *et al*., 1990) were grown at 37°C overnight with aeration in 2 ml of Luria–Bertani (LB) broth. The next morning, 100 ml fresh LB medium was inoculated at a 1:100 dilution with the overnight-grown cultures, and grown at 37°C until the cultures reached mid-log phase growth (A_{600} = 0.7). Total cellular RNA was extracted from 10 ml of cell culture using an RNeasy midi kit (Qiagen, Valencia, CA), following the manufacturer's protocol. The RNA yield and quality were assessed by agarose gel electrophoresis, followed by ethidium bromide staining.

In vitro *rRNA preparation*

The 16S rRNA genes from *E. coli* and *B. subtilis* were PCR amplified from genomic DNA using the bacteria-specific primers 27F (5′-AGAGTTTGATC(A/C)TGGCTCAG-3′) and 1492R (5′-GG(C/T)TACCTTGTTACGACTT-3′), and the resulting PCR products cloned into the pGEM-T Easy vector (Promega). *In vitro* 16S rRNA transcripts from *E. coli* and *B. subtilis*, respectively, were made using the Ribomax *in vitro* transcription system (Promega, Madison, WI). The cloned

rRNA genes were linearized by digestion with *Nco*I to generate a 5′ overhang, and then *in vitro* transcribed according to the manufacturer's protocol. Template DNA was removed using RQ1 RNAse-free DNAse (Promega), followed by phenol–chloroform extraction.

Ribosome preparation

Escherichia coli or *B. subtilis* were grown in 100 ml of LB until the cell cultures reached log-phase growth. The cells were harvested by centrifugation, washed once in a homogenization buffer (20 mM Tris-HCl (pH 7.4), 10.5 magnesium acetate, 100 mM ammonium chloride, 0.5 mM EDTA, and 3 mM β-mercaptoethanol) and then lysed using a French press at 18 000 p.s.i. Cell lysates were subjected to centrifugation at 14 000 r.p.m. in a Beckman microcentrifuge for 30 min at 4°C, after which the supernatants containing intact ribosomes were removed. Ribosomes were pelleted by pipetting the supernatant above a 1.1 M sucrose (in homogenization buffer) cushion and subjecting the samples to centrifugation at 37 500 r.p.m. in a Beckman 70 Ti rotor for 16 h at 4°C. The ribosomal pellet was washed with 70% ethanol, dried and resuspended in RNAse-free water. To remove ribosome-associated proteins, the samples were first fragmented (see below) and then treated with 1 mg ml^{-1} proteinase K for 4 h at 37°C, followed by five rounds of phenol–chloroform extraction. The RNA yield and size were determined by agarose gel electrophoresis, and the loss of ribosome-associated proteins was verified by polyacrylamide gel electrophoresis.

RNA fragmentation

RNA was incubated in a magnesium fragmentation buffer (40 mM Tris acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate) at 95°C for 10 min (de Saizieu *et al.*, 1998). The RNA was subsequently ethanol precipitated, rinsed multiple times in DEPC-treated 70% ethanol, and resuspended in RNAse-free water. The extent of RNA fragmentation was assessed by agarose gel electrophoresis, revealing RNA fragments ranging from 50 to 200 bp in size. The RNA samples were stored at −20°C.

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