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A comparison of DNA fragmentation methods – applications for the biochip technology

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HIGHLIGHTS

- Different ways of DNA fragmentation before labelling and hybridization are studied.
- The ways of enzymatic fragmentation influence on the labelling efficiency.
- DNA fragmented by NEBNext dsDNA Fragmentase is labelled with the greatest efficiency.
- The use of this enzyme increases the sensitivity of biochip-based detection significantly.

Abstract

The efficiency of hybridization signal detection in a biochip is affected by the method used for test DNA preparation, such as fragmentation, amplification and fluorescent labelling. DNA fragmentation is the commonest methods used and it is recognised as a critical step in biochip analysis. Currently methods used for DNA fragmentation are based either on sonication or on the enzymatic digestion. In this study, we compared the effect of different types of enzymatic DNA fragmentations, using DNase I to generate ssDNA breaks, NEBNext dsDNA fragmentase and *SaqAI* restrictase, on DNA labelling. DNA from different *Desulfovibrio* species was used as a substrate for these enzymes. Of the methods used, DNA fragmented by NEBNext dsDNA Fragmentase digestion was subsequently labelled with the greatest efficiency. As a result of this, the use of this enzyme to fragment target DNA increases the sensitivity of biochip-based detection significantly, and this is an important consideration when determining the presence of targeted DNA in ecological and medical samples.

Key words: biochip; DNA fragmentation; SRB; hydrogenase genes; hybridization

1. Introduction

The detection and identification of rare, or low abundant, sequence targets from mixed samples is an important aspect of any environmental analysis, and it is one task that is best performed unambiguously using microarrays. The hybridization of environmental DNA to specific probes

arrayed on a solid support (Kelly, 2009) can be enhanced for the detection of rare sequences by including pre-amplification steps, for either specific genes or whole genomes. Beside the specificity of the probes, the level of biochip sensitivity is influenced by the DNA fragmentation and fluorescent labelling stages prior to hybridization (Gabig-Ciminska et al., 2004). In this study we compared the labelling efficiencies when DNA was digested with DNase I, or NEBNext dsDNA fragmentase or *SaqAI* restrictase, using DNA extracted from 6 species of *Desulfovibrio*. Species of which are renown for being present in environments in low densities.

Sulfate-reducing bacteria (SRB) are anaerobic organisms that play important roles in many biogeochemical processes, and they form regular components of natural and engineered systems at low densities (He et al., 2007). The metabolic capabilities of SRBs are narrow, and these organisms can be detected molecularly by identifying the functional genes for dissimilatory sulfite reductase (*dsr*) and adenosine 5'-phosphosulfate reductase (*apr*). Genes that are directly associated with the reduction of inorganic sulfate (He et al., 2007; Zinkevich, Beech, 2000). Genomic studies of SRB have contributed to an understanding of their basic biochemical mechanisms and the roles they might play in the environment. SRBs play a crucial role in the contamination of petroleum products, resulting in the souring of gas wells over time, and they play a role in the anaerobic corrosion of steel (biocorrosion) (Angel, White, 1995). The interaction with metals is facilitated by the action of hydrogenases, enzymes that catalyse the use of hydrogen gas during metabolism, and the products of these processes accelerate biocorrosion (Caffrey et al., 2007). SRB also attract attention because they can enzymatically reduce and precipitate toxic metals, including U(VI), Cr(VI), Tc(VI) and As(V) and, consequentially, have a role in the bioremediation of metal contaminated environment (Junier et al., 2009). *Desulfovibrio* species have been shown to form metal nanoparticles (Capeness et al., 2015). As a result of their environmental importance and their natural low densities, SRBs are a good choice to assess the processes involved in sequence identification using biochips.

The biochip we constructed comprised oligonucleotide probes for 16S rRNA, and the genes encoding adenosine 5'-phosphosulfate reductase (*apr*) and Ni,Fe periplasmic hydrogenase (small and large subunits). The probe sequences (Table 1) were based on the *in silico* sequence and from experimental results. The nucleotide sequence of the probe **16S_CONS_1** represents the conserved region flanking the variable V3 region of 16S rDNA (Muyzer et al., 1993). This probe enables the detection of most bacteria likely to be present in a bacterial consortium. The

SRB probe covers a fragment of the adenosine-5'-phosphosulfate (APS) reductase gene, and it was used successfully as a PCR primer for the general screening of sulfate-reducing bacteria such as: *Desulfovibrio indonesiensis*, *Desulfovibrio alaskensis*, *Desulfovibrio vietnamensis*, *Desulfovibrio vulgaris*, *Desulfovibrio gigas*, *Desulfovibrio desulfuricans*, *Desulfomicrobium baculatus*, *Desulfococcus multivorans*, *Desulfobulbus propionicus*, *Desulfofrigus fragile*, *Desulfofrigus oceanense*, *Desulfotalea psychrophila*, *Desulfotalea arctica*, *Desulfofada gelida*, *Desulfocinum infernum*, *Desulfotomaculum nigrificans*, *Desulfosporosinus orientis* (Zinkevich, Beech, 2000). This probe will detect a wide range of SRBs, although it cannot distinguish between them. The probes **Dv_HynB** and **Dv_HynA** are designed to specifically recognize the hydrogenase genes *hynA* and *hynB* from *Desulfovibrio spp.* Six *Desulfovibrio* species (*Desulfovibrio magneticus*; *Desulfovibrio gigas*; *Desulfovibrio vulgaris*; *Desulfovibrio alaskensis*; *Desulfovibrio vietnamensis*; *Desulfovibrio indonesiensis*) were used in the design and testing for these probes.

2. Materials and Methods

2.1. The biochip matrix

We have produced two generations of dendrimeric matrix on a microscope slide (Beier, Hoheisel, 1999; Tomalia, 2004), where the acylation and amination reactions have been repeated twice. Polyamine tetraethylenepentamine (TEPA) was used for the first amination step, producing a branched dendrimeric structure. The second amination reaction used diamine 1,4-bis-(3-aminopropoxy)butane (BAPB). The same acylation agent, acryloylchloride, was used in both acylation reactions.

The matrix was activated by the addition of 1 mmol N,N'-disuccinimidylcarbonate (DSC) and 1 mmol diisopropylethyl-amine (DIEA) in 20 ml anhydrous acetonitrile for 4 hrs. DSC is a homobifunctional crosslinking agent and is used for bond formation between the functional group of oligonucleotide probes and the amino group of the dendrimeric structure. Afterwards, the slides were washed with N,N-dimethylformamide (DMF), 1, 2- dichloroethane and dried.

2.2 . Immobilization of oligonucleotide probes onto the activated dendrimeric matrix

The oligonucleotide probes were modified by the addition of a C6 Amine linker at the 5'-end during synthesis. An oligonucleotide probe solution (250 pmol/0.1 μ l) in 1% DIEA was placed onto the activated dendrimeric matrix. Spotting was performed by pins (200 nl/spot) with two replicate spots of each probe being applied to the biochip. The slides were then incubated overnight in a humid chamber at 37°C and subsequently washed with water and ethanol. The surface of the glass slides with the immobilized probes was deactivated by treatment with a solution made of 6-amino-1-hexanol (50 mM) and DIEA (150 mM) in DMF for 2 hours, in order to prevent the binding of the fluorescently labelled DNA with the matrix surface. Finally, the biochips were washed with DMF, acetone, water and dried. The deactivated glass slides with the immobilized probes were then ready to be used in hybridization experiments.

2.3. Cassette construction

The cassette method (Zinkevich et al., 2014) to evaluate biochips was further extended, using a single stranded (*ss*) DNA cassette as the target for hybridization with probes that were coupled to the matrix so that the hybridization capacity of each probes to be evaluated. The *ss*-DNA cassette is a lineal array of sequences complimentary to the studied set of probes. Cy3 fluorescent dye was inserted at the 5'-end of the *ss* cassette during the synthesis. The cassette (83 bases) contained four probe sequences (Supplementary Material), which were synthesized by Bioneer Corporation (Daejeon, Republic of South Korea). The hybridization buffer for the *ss*-DNA cassette was SSARC (4xSSC [600 mM NaCl, 60 mM Na-citrate], 7.2% (v/v) Na-sarcosyl). Hybridization reactions were performed at 45°C for 4 hrs. After hybridization the biochip was washed with 2xSSC + 0.2% SDS for 2 minutes, followed by 0.2xSSC + 0.2% SDS for 2 minutes and finally with 0.2xSSC for 2 minutes at 25°C, and dried by centrifugation.

2.4. DNA preparation for the hybridization and hybridization conditions

DNA from *D. magneticus* RS-1 (DSM 13731) was amplified using an illustra™ GenomiPhi HY DNA Amplification Kit (25-6600-22 GE Healthcare, Life Science, USA), and purified using a PureLink Quick PCR Purification Kit (K3100-01, Invitrogen, USA) following the manufacturer instructions. DNA fragmentation was performed by digesting with one of three different enzymes: NEBNext dsDNA Fragmentase (MO348S, New England BioLabs, USA); FastDigest *Saq*AI (FD2174, Thermo Scientific, USA); or DNaseI from the ULYSIS® Alexa Fluor®546 Nucleic Acid Labeling Kit (U21652, Molecular Probes, USA) following the manufacturer

instructions. The digestion conditions for each enzyme were selected to form a range of fragment sizes from 50 to 200 bp. DNA fragmentation was estimated by electrophoresis on 1.5% agarose gel in 1xTAE (40 mM Tris-acetate, 1 mM Na₂EDTA) buffer, and visualized by Ethidium bromide staining. The fragmented DNA was labelled by covalent bonding it to the fluorescent dye Alexa Fluor®546 (U21652, Molecular Probes, USA), according to the manufacturer protocol. The labelled DNA was purified using Bio-Rad Micro Bio-Spin® P-30 columns (732-6202, Bio-Rad Laboratories, USA). The labelling efficiency and DNA concentration were calculated according to the manufacturer protocol. The purified DNA was precipitated by adding 10 volumes of 2% LiClO₄ in acetone for at least 30 min at -20°C, and sedimented by centrifugation at 15,000 rpm for 20 min at 4°C, then washed with acetone and dried. The hybridization buffer for the amplified, fragmented and labelled bacterial DNA was 1 M GuSCN (guanidine thiocyanate), 5 mM EDTA, 50 mM HEPES (pH 7.5), and 0.2 mg/ml BSA (bovine serum albumin). The test DNA, containing 160 pmol Alexa546, was heated at 95°C for 5 min, chilled immediately on ice for 2 min, and hybridized at 25°C for 4 hrs. After hybridization the biochip was washed with 4XSSC + 7.2% Sarcosyl for 2 min, dried by centrifugation in a Microarray High Speed Centrifuge (ArrayIt, USA), and visualized using a Portable Imager 5000 (Aurora Photonic, USA) with 532 nm green Laser and 580 nm filter. The signal intensity of each point on the biomatrix was calculated using MicroChip Imager software (Aurora Photonics, USA).

DNA samples from *D. magneticus* RS-1 DSM 13731, *Desulfovibrio vulgaris* str. Hildenborough NCIMB 8303, *Desulfovibrio gigas* DSM 1382, *Desulfovibrio alaskensis* AL1 NCIMB 13491, *Desulfovibrio vietnamensis* DSM 10520, and *Desulfovibrio indonesiensis* NCIMB 13468 were amplified using an illustra™ GenomiPhi HY DNA Amplification Kit, and purified using a PureLink Quick PCR Purification Kit. The amplified and purified DNA was fragmented by NEBNext dsDNA Fragmentase, and labelled with Alexa Fluor®546 dye.

3. Results and Discussion

3.1. Optimization of the experimental conditions

The influence of DNA fragmentation methods on hybridization signals in the designed biochip was evaluated. Before this could be achieved it was necessary to ensure that each probe produced

similar hybridization signals under standard conditions. The cassette method (Zinkevich et al., 2014) was used to estimate the hybridization capacity of the four probes present in the biochip. All probes show a similar hybridization capacity (Fig. 1).

The products of enzymatic fragmentation differed according to which method was used. DNase I digested DNA produced ssDNA breaks randomly, whereas a *SaqAI* restrictase, which has a specific four-base pair recognition sequence, digestion produced dsDNA breaks with sticky ends, and NEBNext dsDNA Fragmentase generated dsDNA breaks with blunt ends. DNA from *Desulfovibrio magneticus* RS-1 DSM 13731, the genome of which has been completely sequenced (Nakazawa et al., 2009) was used to standardize the condition for enzymatic fragmentation. DNA was digested with each selected enzyme, and labelled with the fluorescent dye Alexa Fluor®546, after which the labelling efficiency was determined for all DNA fragmented forms. DNA treated with NEBNext dsDNA Fragmentase was labelled with a greater efficiency compared to the DNA fragmented by DNase I and *SaqAI* (Table 2), with the density of the dye molecules per nucleotide base being 3-4 times higher in these molecules. Figures 2A and 2B show the results when *D. magneticus* DNA was fragmented by different enzymes and subsequently hybridized to the biochip. The DNA fragments larger than 1000 bp can aggregate during the labeling and therefore effect on its efficiency. To avoid it the conditions for all enzymes were optimised to produce a range of fragments from 50 to 200 bp (Figures 2A). The amount of dye molecule per nucleotide base was normalised before hybridization. All of the probes displayed the same hybridization pattern, independent of the way the DNA was fragmented, when the quantity of fluorescent dye in the DNA molecule was regarded. This indicated that the DNA labelled after Fragmentase digestion had greater hybridization sensitivity because more molecules would have received label, enabling a signal to be produced with less DNA.

3.2. Validation of DNA fragmentation

DNA samples from six *Desulfovibrio* spp. (*D. vulgaris* str. Hildenborough; *D. gigas*; *D. alaskensis* AL1; *D. vietnamensis*; *D. indonesiensis*, *D. magneticus* RS-1) were fragmented using NEBNext dsDNA Fragmentase and subsequently labeled with Alexa Fluor®546. The same labeling efficiency has been observed for all DNA (Table 2). The fragmented and labeled DNA was then used in hybridization reactions on the biochip (Fig. 3). The complete genome sequence

was known for three of the *Desulfovibrio* species (*D. magneticus* RS-1, *D. vulgaris* str. Hildenborough, and *D. gigas*), whereas only the 16S rRNA gene sequences were known for *D. alaskensis* AL1, *D. vietnamensis*, and *D. indonesiensis*. The sequence of the probe **16S_CONS_1** was present in all of the DNA molecules studied. Likewise, the presence of the **SRB** probe sequence was shown experimentally to be in all of the DNA molecules studied, including *D. alaskensis* AL1, *D. vietnamensis*, and *D. indonesiensis* (Zinkevich, Beech, 2000). Figures 3A and B show the results obtained when six of the *Desulfovibrio* DNA samples were hybridized with the probes **16S_CONS_1** and **SRB** on the biochip. The same signal levels were observed for all of the hybridization reactions.

The probe **Dv_HynB** (CACCCCTGCATCGGCTGCAG) was derived from *hynB* gene of *D. magneticus* RS-1, which encoded the small subunit of periplasmic [NiFe] hydrogenase (Table 1). The same sequence was also present in the *hynB* gene of *D. vulgaris* str. Hildenborough. The results of hybridization to this probe are shown in Fig. 3C. DNA from *D. vulgaris* and *D. magneticus* RS-1 gave the same hybridization signals against probe **Dv_HynB**. The one nucleotide difference in the *hynB* gene sequence of *D. gigas* (CACCCCTGCATCGCCTGCAG) resulted in a significant decrease in signal intensity for this species. This was expected as it has been stated previously that single nucleotide differences can result in reduced signal responses when nucleotide-based microarray are used (Bavykin et al., 2008). The observation that similar hybridization signals for probe **Dv_HynB** with the DNA from *D. alaskensis* AL1 were obtained indicated that this gene was present in the genome with an identical sequence, which was expected as it is present in strain G20 of this species (Table 1). Similar hybridization signals were obtained for DNA from *D. vietnamensis* and *D. indonesiensis*, *D. vulgaris* and *D. magneticus* RS-1, indicating that identical sequences to probe **Dv_HynB** exist in their genomes.

The **Dv_HynA** probe sequence was derived from the *hynA* gene of *D. magneticus* RS-1, which encodes the large subunit of periplasmic [NiFe] hydrogenase. The same sequence was present in the DNA of *D. gigas* (Table 1), in contrast to the **Dv_Hyn B** sequence. The hybridization of genomic DNA against this probe is shown in Fig. 3D. The intensity of hybridization signal was the same with DNA from *D. magneticus*, *D. gigas*, *D. vietnamensis*, and *D. indonesiensis*, but was absent when DNA from *D. vulgaris* str. Hildenborough was used. This is consistent with the notion that the **Dv_HynA** probe sequence is absent from the *hynA* gene of *D. vulgaris* str. Hildenborough (Heidelberg et al., 2004). A lower intensity hybridization signal

was observed when the DNA from *D. alaskensis* AL1 was used, compared to that produced when the DNA from *D. magneticus* RS-1 and *D. gigas* were used. This could be because a difference exists in the probe **Dv_HynA** sequence for *D. alaskensis* AL1. The genomes of *D. vietnamensis*, *D. indonesiensis* are not completely sequenced, but their DNA generated the same intensity hybridization signals as those from *D. gigas* and *D. magneticus* RS-1, suggesting that they share the probe sequence. The results of this study suggest that 4 out of 6 *Desulfovibrio* species shared the **Dv_HynA** probe sequence in their genomes.

4. Conclusion

Fragmentation is a crucial step in the preparation of DNA prior to labelling and the subsequent signal visualization after hybridization. The data presented in this study shows that the efficiency of labelling depends on the type of DNA fragmentation. NEBNext dsDNA Fragmentase DNA digestion produced fragmented DNA that was labelled with the greatest efficiency. This well labelled DNA allowed the reliable identification of nucleotide sequences in hybridization experiments and had the advantage of enhanced sensitivity, which is particularly important for detection of target DNA in environmental and medical samples.

Conflict of interest

The authors declare that they have no conflict of interest

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FIGURE CAPTIONS

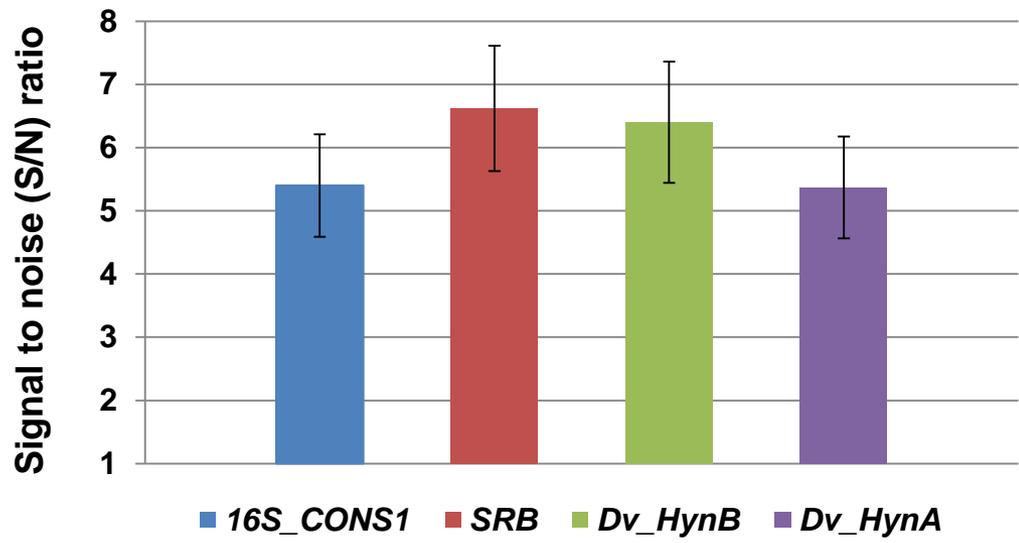
Figure 1. Quantitative analysis of cassette hybridization with biochip. The cassette shows the hybridization signals for four probes (*16S_CONS1*, *SRB*, *Dv_HynB* and *Dv_HynA*). The results of the hybridization are presented as a signal to noise (S/N) ratio. The data presented are mean values \pm SD from three separate sets of experiments.

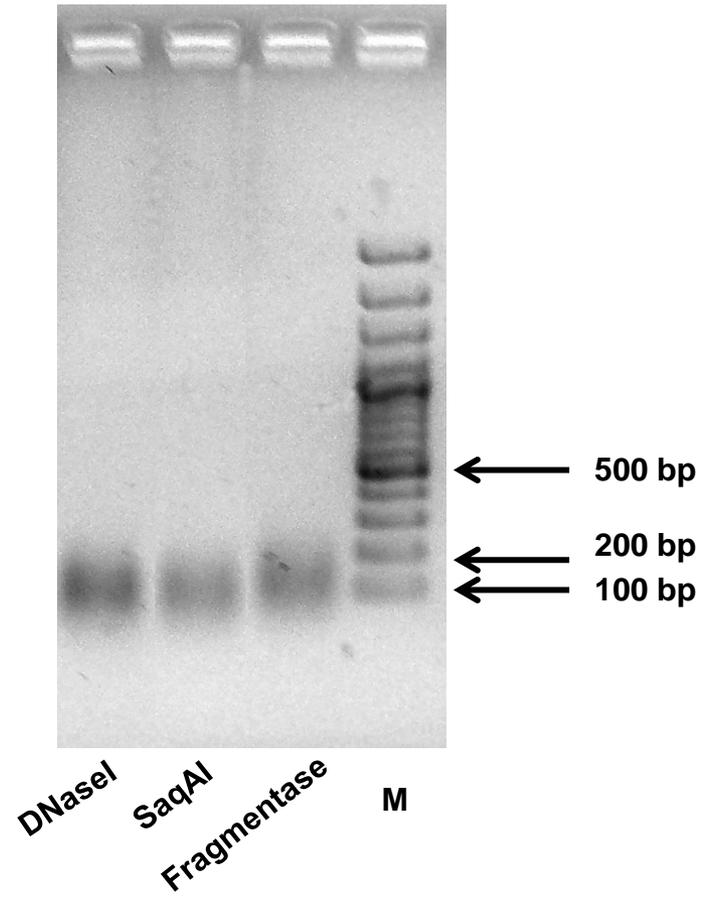
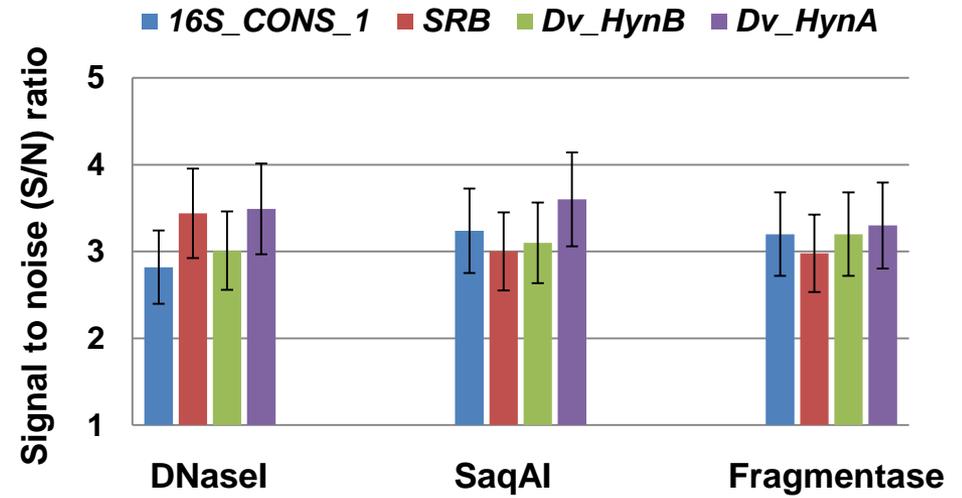
Figure 2. Quantitative analysis of *D. magneticus* RS-1 DNA hybridization with biochip.

Panel (A) shows the result of DNA fragmentation by three different enzyme treatments: DNaseI, restrictase *SaqAI*, or NEBNext dsDNA Fragmentase. Lane M: GeneRuler 100 bp. Panel (B) shows the result of the hybridization. The data presented are mean values \pm SD from three separate sets of experiments.

Figure 3. Quantitative analysis of hybridization of six *Desulfovibrio* species DNAs using a biochip. DNA was fragmented by NEBNext dsDNA Fragmentase. Panel (A) shows the result of hybridization of the fragmented DNA with the probe *16S_CONS1*. Panel (B) shows the result of hybridization of the fragmented DNA with the probe *SRB*. Panel (C) shows the result of

hybridization of the fragmented DNA with the probe *Dv_HynB*. Panel (D) shows the result of hybridization of the fragmented DNA with the probe *Dv_HynA*. The results of the hybridization are presented as a signal to noise (S/N) ratio. The data presented are mean values \pm SD from three separate sets of experiments.



A**B**

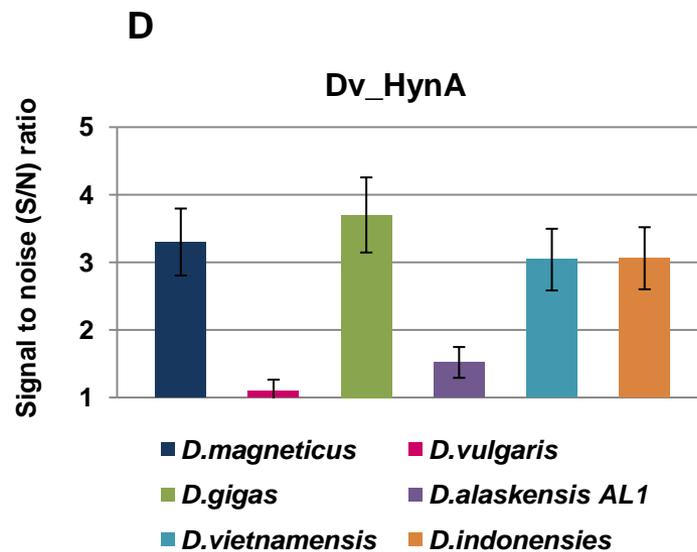
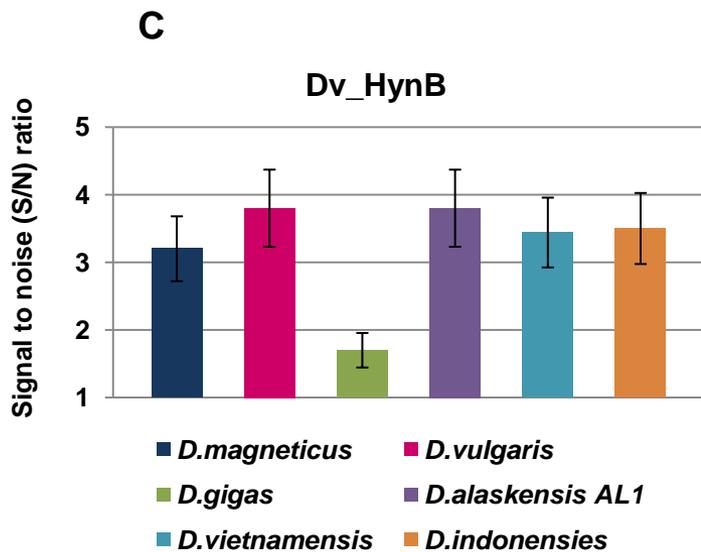
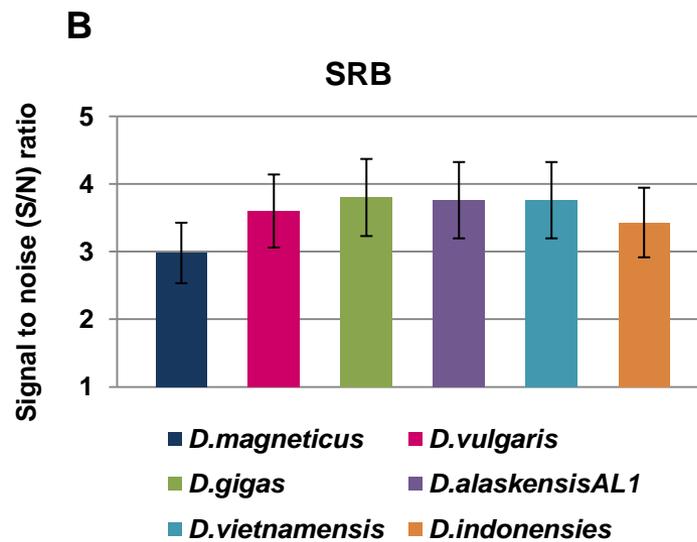
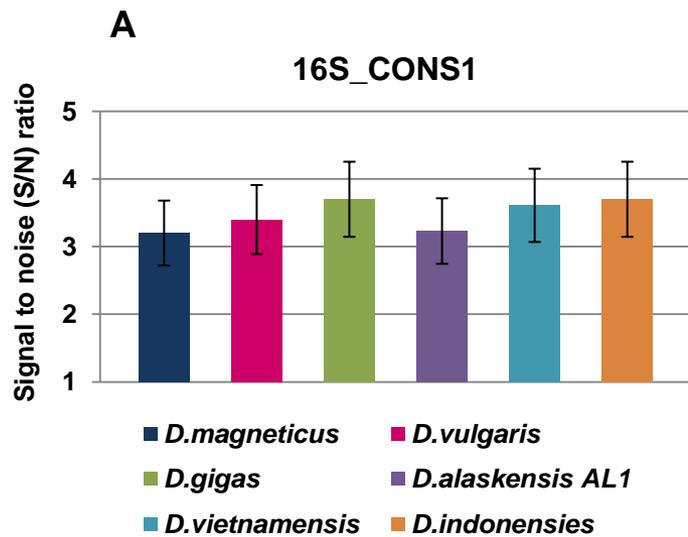


Table 1. Characteristics of the probes used in this study

Annotation	Probe name	DNA sequence 5'→3'	Gene Bank
16S rRNA	16S_CONS_1	CCTACGGGAGGC AGCAG	AP010904.1 AE017285.1
Adenosine 5'-phosphosulfate reductase (<i>apr</i>)	SRB	CCAGGGCCTGTC CGCCATCAATAC	AE017285.1
Periplasmic [NiFe] hydrogenase, small subunit (<i>hynB</i>)	Dv_HynB	CACCCCTGCATC GGCTGCAG	AP010904.1 <i>Desulfovibrio magneticus</i> RS-1
Periplasmic [NiFe] hydrogenase, small subunit (<i>hynB</i>)	Dv_HynB	CACCCCTGCATC GGCTGCAG	AE017285.1 <i>Desulfovibrio vulgaris</i> str. Hildenborough
Periplasmic [NiFe] hydrogenase, small subunit (<i>hynB</i>)	Dv_HynB	CACCCCTGCATC GGCTGCAG	CP000112.1 <i>Desulfovibrio alaskensis</i> G-20
Periplasmic [NiFe] hydrogenase, small subunit (<i>hynB</i>)	Dv_HynB	CACCCCTGCATC GCCTGCAG	CP006585.1 <i>Desulfovibrio gigas</i>
Periplasmic [NiFe] hydrogenase, large subunit (<i>hynA</i>)	Dv_HynA	GCGACGCCCAGC ACT TCACCCA	AP010904.1 <i>Desulfovibrio magneticus</i> RS-1
Periplasmic [NiFe] hydrogenase, large subunit (<i>hynA</i>)	Dv_HynA	GCGACGCCCAGC ACTTCACCCA	CP006585.1 <i>Desulfovibrio gigas</i>
Periplasmic [NiFe] hydrogenase, large subunit (<i>hynA</i>)	Dv_HynA	N/I*	AE017285.1 <i>Desulfovibrio vulgaris</i> str. Hildenborough

*N/I - The sequence GCGACGCCCAGCACTTCACCCA is not identified in the *Desulfovibrio vulgaris* str. Hildenborough (NCIMB 8303) genome.

Table 2. Labelling efficiency after different types of fragmentation

DNA	Type of fragmentation	Labelling ratio*
<i>D. magneticus</i> RS-1	DNaseI	19 ± 2
	<i>SaqAI</i>	26 ± 2
	Fragmentase	78 ± 6
<i>D. vulgaris</i> str. Hildenborough	Fragmentase	75 ± 6
<i>D. gigas</i>	Fragmentase	76 ± 5
<i>D. alaskensis</i> AL1	Fragmentase	77 ± 6
<i>D. vietnamensis</i>	Fragmentase	79 ± 7
<i>D. indonesiensis</i>	Fragmentase	71 ± 5

* pmol Dye/μg DNA ratio for the labelled nucleic acid

Each value is the mean ± SD from three separate sets of experiments