emi_575.fm Page 1 Friday, February 13, 2004 4:23 PM

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Suppressive subtractive hybridization as a tool for identifying genetic diversity in an environmental metagenome: the rumen as a model

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Summary

Molecular techniques previously used for genome comparisons of closely related bacterial species could prove extremely valuable for comparisons of complex microbial communities, or metagenomes. Our study aimed to determine the breadth and value of suppressive subtractive hybridization (SSH) in a pilot-scale analysis of metagenomic DNA from communities of microorganisms in the rumen. Suppressive subtractive hybridization was performed using total genomic DNA isolated from rumen fluid samples of two hay-fed steers, arbitrarily designated as tester or driver. Ninety-six subtraction DNA fragments from the tester metagenome were amplified, cloned and the DNA sequences were determined. Verification of the isolation of DNA fragments unique to the tester metagenome was accomplished through dot blot and Southern blot hybridizations. Tester-specific SSH fragments were found in 95 of 96 randomly selected clones. DNA sequences of subtraction fragments were analysed by computer assisted DNA and amino acid comparisons. Putative translations of 26 (32.1%) subtractive hybridization fragments exhibited significant similarity to Bacterial proteins, whereas 15 (18.5%) distinctive subtracted fragments had significant similarity to proteins from Archaea. The remainder of the subtractive hybridization fragments displayed no similarity to GenBank sequences. This metagenomic approach has exposed an unexpectedly large difference in Archaeal community structure between the rumen microbial populations of two steers fed identical diets and housed together. 16S rRNA dot blot hybridizations revealed similar proportions of Bacteria and Archaea in both rumen samples and suggest that the differences uncovered by SSH are the result of varying community structural composition. Our study demonstrates a novel approach to comparative analyses of environmental microbial communities through the use of SSH.

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Introduction

Metagenomics addresses the collective genetic structure and functional composition of a microbial environmental sample without the bias or necessity for culturing the microorganisms from the community in question. An extensive suite of molecular based approaches developed over the past decade have enabled the study of these uncultured microorganisms and are the direct outcome of the use of small subunit ribosomal RNA (SSU rRNA) targets which have been widely utilized to study the bacterial diversity, community structure, and microbial interactions within these microbial ecosystems (Pace, 1997; Theron and Cloete, 2000; Torsvik and Øvreås, 2002). Additionally, molecular fingerprinting strategies such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and phospholipids fatty acid (PLFA) analysis have been utilized to analyse microbial community composition and monitor alterations in community structure from complex environments such as the gastrointestinal tract (Simpson *et al.*, 1999; Zoetendal *et al.*, 2002), marine sediments (Braker *et al.*, 2001) and oil-contaminated soils (Bundy *et al.*, 2002) respectively. Community genome complexity and size have also been estimated by analysing the reassociation of genomic DNA from environmental samples (Torsvik *et al.*, 1998). Studying the functionality of these metagenomes has been approached by the use of fluorescence *in situ* hybridization (FISH) in combination with microautoradiography, which permits both the detection of microbial populations and the measurement of substrate utilization within a microbial community (Gray *et al.*, 2000; Nielsen *et al.*, 2003). Investigators have also used non-SSU rDNA targets in a molecular metabolic ecology (MME) approach for following the populations of microorganisms in complex communities (Deplancke *et al.*,

emi_575.fm Page 2 Friday, February 13, 2004 4:23 PM

2000). Furthermore, DNA microarray technology has been applied to determine patterns of metabolic gene expression within mixed microbial systems (Dennis *et al*., 2003; Taroncher-Oldenburg *et al*., 2003). Although these methods provide a wealth of information about microbial community structure, they are limited to a subset of identifying sequences.

Thus, metagenomic studies are now beginning to take advantage of the plethora of complete genome sequences (Doolittle, 1997; Fraser *et al*., 2000) and the associated tools to discover novel genes and survey the structure and function of microbial communities. Construction of metagenomic libraries using bacterial artificial chromosome (BAC) vectors (Béjà *et al*., 2000; Rondon *et al*., 2000) is a direct route to gaining insight into the functional nature of mixed microbial samples. In particular, Béjà and co-workers uncovered large genomic fragments (≈80 kb) from a diverse population of largely uncultivated microorganisms and assigned functional properties to many of the genes on a 60 kb sequence from an Archaeal clone (Béjà *et al*., 2000). Specifically, an association of three genes possibly involved in a novel membrane-associated proteolytic system was observed (Béjà *et al*., 2000). Direct cloning approaches have also been applied to construct soil DNA libraries to facilitate screening for specific enzyme activity (Henne *et al*., 1999; Majerník *et al*., 2001) and to bio-prospect for novel antibiotics (Gillespie *et al*., 2002). The sequencing and assembly of these large gene insert libraries have also been hypothesized to lead to reconstruction of a nearly complete microbial genome (DeLong, 2002).

Complementary and less expensive methods to compare genomes from individual microbes have been utilized in comparative genomic studies. Suppressive subtractive hybridization (SSH) is one such approach, which has been utilized to compare the genomic content of closely related species of bacteria (Akopyants *et al*., 1998; Mavrodi *et al*., 2002; Nesbø *et al*., 2002; Smoot *et al*., 2002). Strain-specific DNA fragments can be isolated by this method and compared to sequences of known genes or proteins, thus linking functional data to unique DNA fragments within the compared genomes. For instance, Nesbø and co-workers used SSH to examine the genomic diversity in *Thermotoga maritima* and assigned functional information to many DNA fragments including a large proportion involved in sugar transport and degradation, implicating important differences in polysaccharide utilization between *Thermotoga* strains (Nesbø *et al*., 2002). Genetic differences between closely related pathogens such as virulence plasmid-related sequences, prophage sequences, and pathogenicity islands have also been identified through the use of this technique (Bogush *et al*., 1999; Emmerth *et al*., 1999; Winstanley, 2002)**.** The utilization of this comparative analysis has provided a unique perspective on the genetic diversity and functionality of closely related genomes.

Here, we describe the application of SSH as a comparative method to examine the microbial diversity (i.e. species composition) and functional differences (i.e. gene composition) in the genomic content of two different environmental communities. Our model system is the microbial community of the rumen and therein we aim to validate the use of SSH for analysing very complex microbial communities. Specific rumen samples were chosen for this study because our group had previously examined these samples in phylogenetic studies using DGGE and SSU rDNA libraries (Kocherginskaya *et al*., 2001). Moreover, the Bacterial and Archaeal diversity and functionality within the microbial community of the rumen have long been the foci of rumen microbiologists (Hungate, 1966; Hespell *et al*., 1996) and there is a considerable knowledge base from both classical culture based methods as well as the more current molecular approaches (Raskin *et al*., 1996; White *et al*., 1999). We present data that validates the use of SSH to isolate DNA fragments present in one rumen sample, but absent from another. DNA of these subtracted samples were cloned, the DNA sequences determined, and analysed by computer assisted DNA and amino acid comparisons. Subtractive hybridization fragments of both Bacterial and Archaeal origin were identified, demonstrating that this technique provides a powerful complementary approach to current methods for the metagenomic structural analysis of complex microbial environments.

Results

Suppressive subtractive hybridization of rumen microbial metagenomes

Suppressive subtractive hybridization was carried out between the microbial metagenomes of two rumen community DNA samples from animals fed an identical hay diet in order to examine the utility of this technique for the isolation of DNA unique to an environmental metagenome sample. Rumen metagenome samples were arbitrarily assigned as tester (#256, animal number) or driver (#218, animal number). Ninety-six DNA fragments between 100 and 1 500 bp in size were isolated from tester rumen sample #256 using SSH. The SSH technique displayed a high level of efficiency as measured by dot blot hybridizations. Ninety-five out of 96 subtracted, cloned DNA fragments hybridized to the tester metagenomic DNA probe, whereas only one subtraction fragment (C10) also hybridized to the driver metagenomic DNA probe (Fig. 1A). Differences in the intensity of dot blot hybridization signals were observed (i.e. Fig. 1A, part a, fragments D01 and D02), and this may be the result of unequal membrane loading concentrations or variations in the G/C content of

emi_575.fm Page 3 Friday, February 13, 2004 4:23 PM

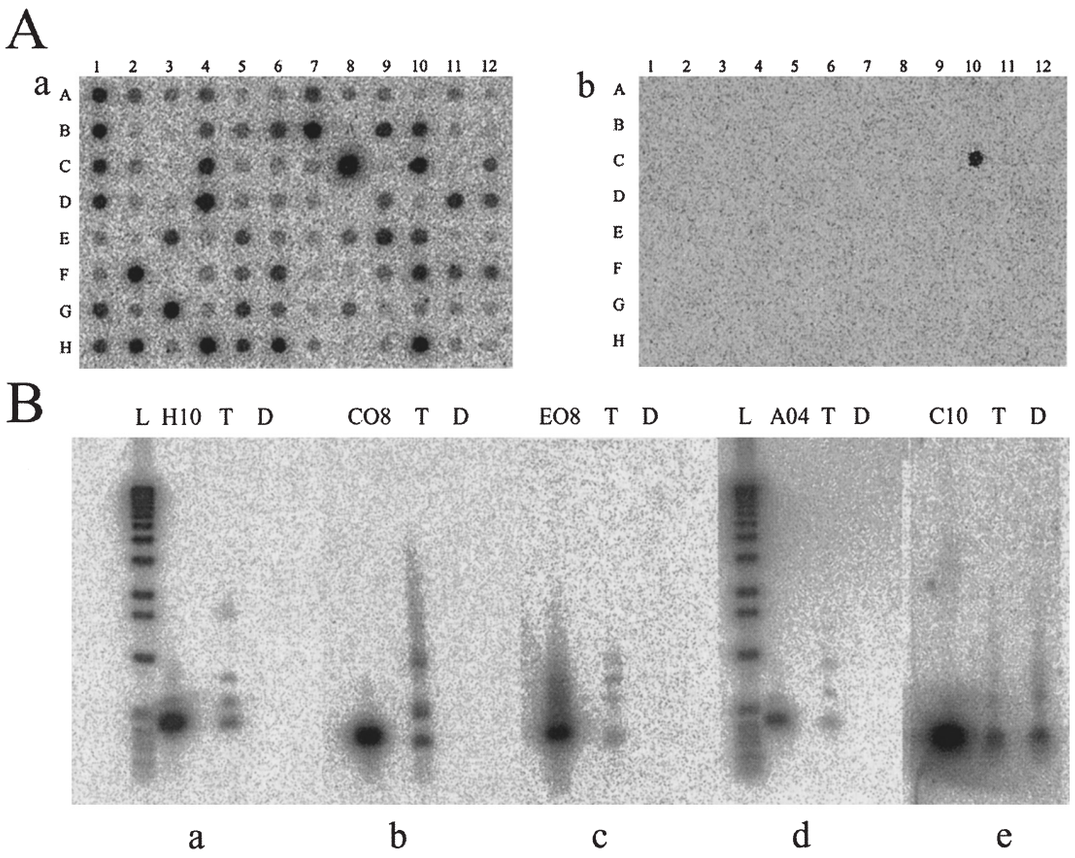

Fig. 1. Hybridization analysis of randomly selected clones from the subtracted library of rumen metagenome sample #256.
A. PCR amplicons from 96 randomly selected clones were transferred to nylon membranes with a dot blot manifold and hybridized with ^{32}P-labelled genomic DNA from the tester metagenome (a) or the driver metagenome (b).
B. Southern blot hybridizations of five clones. Subtracted clones and *Alu* I-digested genomic DNA from both tester (T) and driver (D) were electrophoresed using agarose gels, transferred to nylon membranes and hybridized with respective ^{32}P-labelled subtracted clones. A 1 kb DNA standard ladder (L) (Invitrogen) is also shown. Clones H10 (a), C08 (b), E08 (c), and A04 (d) were randomly chosen. Clone C10 (e) was chosen as a positive control probe to confirm the dot blot hybridization results.

subtraction fragments. The differences in hybridization signals may also be indicative of the relative gene dose in the complex DNA metagenome assembly used as the probe.

Southern blot analysis was used with selected subtraction fragments to further confirm the specificity of SSH for isolating DNA sequences unique to the tester metagenome, by means of demonstrating the presence of subtractive DNA fragments in the tester metagenome, and the absence of these fragments in the driver metagenome. Four randomly selected, SSH clones were used to probe *Alu I* digests of tester and driver metagenomes. Subtraction fragment C10, which hybridized to both tester and driver metagenomes, was also chosen as a control subtraction DNA fragment. Southern blot analysis using the four randomly selected tester-specific subtractive DNA fragments as probes revealed hybridization signals of the appropriate size in the tester DNA, but showed no hybridization signals in the driver DNA (Fig. 1B, a–d). Hybridization signals were detected in both the tester and driver DNAs when Southern blots were probed with clone C10 (Fig. 1B, e). The appearance of multiple bands in the tester digest lanes (or in tester and driver DNAs; Fig. 1B, e) may be attributable to naturally occurring restriction fragment length polymorphisms, indicating the presence of the subtractive DNA fragment in multiple species within the microbial community. Additionally, the multiple hybridization signals may have resulted from incomplete restriction endonuclease digestion caused by methylated or otherwise protected DNA. Both of these situations are plausible because of the complexity of rumen community DNA. Nonetheless, these results support the dot blot hybridization results, and suggest that SSH can be used to examine genetic diversity of complex microbial communities through the isolation of DNA fragments present in only one community.

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emi_575.fm Page 4 Friday, February 13, 2004 4:23 PM

Sequence analyses and protein similarity matches of subtracted clones from rumen sample #256

DNA sequence analysis of the 96 subtraction fragments revealed 81 unique DNA sequences (15.6% redundancy). Computer-assisted DNA and amino acid comparisons of the subtractive hybridization fragments uncovered significant similarities to proteins from Bacterial and Archaeal domains of life as defined by Woese *et al*. (1990) (Table 1). However, 39 (48.1%) subtractive hybridization fragments had no significant match with known sequences in these databases. The relative percentages of identity matches found within each domain of life are summarized in Fig. 2.

Sequences from 26 (32.1%) subtractive hybridization fragments exhibited similarity to Bacterial proteins. The database of Clusters of Orthologous Groups of proteins (Tatusov *et al*., 2001) was utilized as an aid in identifying functions associated with many of the proteins that had significant matches to our subtraction fragments. Of these

Table 1. Rumen sample #256-specific clones with significant protein matches in The National Center for Biotechnology Information protein databases.

Domain and clones	Size, bp	First BLASTX hit; accession no.	Organism	Expect value[a]
Bacteria				
D05	1182	putative DNA polymerase, Bacteriophage SPBc2, NP_046685.1	*Bacillus subtilis*	1.00E-60
A01	929	ATP-dependent DNA helicase recG, NP_603098.1	*Fusobacterium nucleatum*	9.00E-46
A11	711	hypothetical protein, ZP_00061482.1	*Clostridium thermocellum*	1.00E-44
F01	738	hypothetical protein, ZP_00130950.1	*Desulfovibrio desulphuricans* G20	1.00E-39
B12	609	putative oxidoreductase, NP_416641.1	*E. coli* K12	5.00E-42
F10	965	hypothetical protein, ZP_00060522.1	*Clostridium thermocellum*	1.00E-36
C01	1223	putative glycosyltransferase CpsIVI, AAK43612.1	*Streptococcus agalactiae*	4.00E-35
D02	925	surfactin synthetase, NP_388230.1	*Bacillus subtilis*	2.00E-31
E05, F05	701	hypothetical protein, ZP_00129983.1	*Desulfovibrio desulphuricans* G20	4.00E-22
H02, H06	1496	unknown protein, NP_713928.1	*Leptospira interrogans*	2.00E-21
B06	566	hypothetical protein, ZP_00098599.1	*Desulfitobacterium hafniense*	7.00E-21
C12	566	transposase 01, NP_240989.1	*Bacillus halodurans*	4.00E-16
E03, G03	526	transposase, NP_623942.1	*Thermoanaerobacter tengcongensis*	1.00E-14
G07	779	hypothetical protein, ZP_00097387.1	*Desulfitobacterium hafniense*	1.00E-14
F03	485	hypothetical protein, ZP_00060061.1	*Clostridium thermocellum*	6.00E-13
H11	587	hypothetical protein, ZP_00060316.1	*Clostridium thermocellum*	1.00E-12
D12	582	unknown, NP_768825.1	*Bradyrhizobium japonicum*	2.00E-12
C08	331	transposase, S43117	*Leptospira borgpetersenii*	2.00E-09
D01, E01	1229	hypothetical protein, NP_308569.1	*E. coli* O157:H7	6.00E-09
D03	913	lysyl-tRNA synthetase, NP_488111.1	*Nostoc* sp. PCC7120	3.00E-08
A07, B07, B08	621	unknown, yme, NP_059777.1	*Agrobacterium tumefaciens*	3.00E-06
H12	1287	probable collagen adhesin, NP_150050.1	*Clostridium perfringens*	5.00E-06
G08	818	hypothetical protein, ZP_00062228.1	*Clostridium thermocellum*	1.00E-05
C09	975	saliva interacting protein precursor, 1616231 A	*Streptococcus mutans*	1.00E-05
G06	976	hypothetical protein, ZP_00060139.1	*Clostridium thermocellum*	2.00E-05
C07	1256	surface protein (LPXTG motif), NP_470145.1	*Listeria innocua*	5.00E-05
Archaea				
F09	852	anaerobic ribonucleotide triphosphate reductase, NP_276652.1	*Methanothermobacter thermautotrophicus*	1.00E-123
C02, B09	893	DNA dependent RNA polymerase, subunit A, NP_276181.1	*Methanothermobacter thermautotrophicus*	1.00E-120
H09	767	conserved protein, NP_275963.1	*Methanothermobacter thermautotrophicus*	9.00E-78
B01	1158	YI48_METSM Insertion Element ISM1, 43.8 KD, P22344	*Methanobrevibacter smithii*	2.00E-75
E02	1486	conserved protein, contains ferredoxin domain, NP_276495.1	*Methanothermobacter thermautotrophicus*	3.00E-64
D06	619	diphthine synthase, NP_276980.1	*Methanothermobacter thermautotrophicus*	6.00E-59
D07	749	5-methylcytosine-specific restriction enzyme McrB related protein, NP_275644.1	*Methanothermobacter thermautotrophicus*	1.00E-55
H03	895	3-dehydroquinate dehydratase, NP_275709.1	*Methanothermobacter thermoautotrophicus*	1.00E-49
A06	764	unknown, NP_275382.1	*Methanothermobacter thermautotrophicus*	2.00E-33
E06	995	phosphoribosyl-AMP cyclohydrolase, NP_275388.1	*Methanothermobacter thermautotrophicus*	2.00E-32
G09	393	O-linked GlcNAc transferase, NP_276697.1	*Methanothermobacter thermautotrophicus*	2.00E-22
G12	486	multidrug transporter homolog, NP_275247.1	*Methanothermobacter thermautotrophicus*	3.00E-19
E11	1037	amidase, NP_276610.1	*Methanothermobacter thermautotrophicus*	3.00E-10
C05, D04	622	cysteine proteinase, NP_632235.1	*Methanosarcina mazei* Goel	3.00E-09
F04	777	surface layer protein B, NP_635097.1	*Methanosarcina mazei* Goel	4.00E-09

a. Expectation values of 1.00E-05 and below were considered as significant matches during database searches.

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emi_575.fm Page 5 Friday, February 13, 2004 4:23 PM

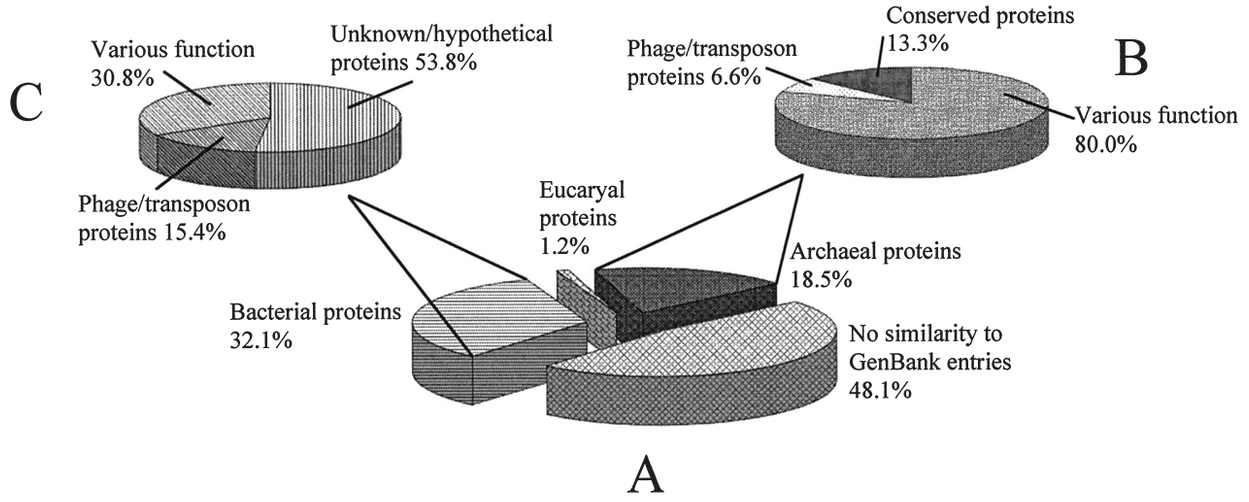

Fig. 2. Distribution of protein similarity matches from the subtractive hybridization library of rumen metagenome sample #256 classified by domain of life and function. The centre pie chart (A) organizes SSH fragments according to the domain of their highest sequence similarity matches. Classifications of subtraction fragments by function are depicted for fragments with highest sequence similarity to Archaeal proteins (B) and to Bacterial proteins (C).

Bacterial similarity matches, 14 DNA fragments resembled open reading frames (ORFs) of hypothetical or unknown function. Subtraction fragments A01 and D05 resembled putative genes involved in DNA replication, recombination and repair. Clones resembling genes for putative Bacterial proteins involved in energy production and conversion (B12), cell envelope biogenesis (C01), and translation, ribosomal structure and biogenesis (D03), were also found. One DNA fragment, D02, showed protein sequence similarity to a surfactin synthetase (subunit I) from *Bacillus subtilis*. This protein functions as part of a multienzyme complex to catalyse the synthesis of a powerful biosurfactant with antimicrobial activities (Vollenbroich *et al*., 1994; Symmank *et al*., 2002). Also, half (13/26 or 50.0%) of the observed Bacterial protein similarity matches were from low-G/C Gram-positive organisms. This result is consistent with DNA sequence data collected from previous analysis of the microbial community SSU rDNA in our rumen samples (Kocherginskaya *et al*., 2001). In addition, proteins from one particular low-G/C, Gram-positive organism, the highly cellulolytic *Clostridium thermocellum*, provided the closest similarity matches for six (7.4%) of the subtractive hybridization fragments.

Fifteen (18.5%) distinctive subtraction fragments had significant similarity to proteins from Archaeal representatives. These proteins encompassed a variety of cellular functions including amino acid transport and metabolism (E06 and H03), transcription (C02), and translation, ribosomal structure and biogenesis (D06). In particular, fragment F09 showed a high level of sequence similarity to an anaerobic ribonucleotide triphosphate reductase from *Methanothermobacter thermautotrophicus*. The Archaeal enzyme, involved in the reduction of nucleotide diphosphates to nucleotide triphosphates, has distinguishing characteristics that set it apart from its Bacterial counterparts such as susceptibilities to S-adenosylmethionine, oxygen, and azide, and an independence from coenzyme B12, manganese, and iron (Sprengel and Follmann, 1981; Sze *et al*., 1992). Moreover, it has been suggested that the Archaeal ribonucleotide reductase may have a different activation mechanism than Bacterial ribonucleotide reductases (Sze *et al*., 1992).

The redundancy observed in the DNA sequence analysis of subtraction fragments exhibited no apparent trends with regard to fragment length and similarity match characteristics. Redundant sequences ranged in length from 350 bp to nearly 1500 bp. Additionally, examination of redundant clones paralleled analysis of the unique subtraction fragments, as slightly less than half (7/15) exhibited no similarity to existing database entries, whereas 53% (8/15) displayed significant similarity to proteins from Bacteria or Archaea. Correlation between redundancy and protein function was not observed. In light of these observations, redundant sequences are likely an experimental artifact and of no ecological significance.

Analysis of 16S rRNA from rumen samples #218 and #256

The 16S rRNAs from rumen samples #218 and #256 were probed with universal (S-*-University-1392-a-A15), Bacterial-specific (S-D-Bact-0338-a-A-18), and Archaeal-specific (S-D-Arch-0915-a-A-20) probes to characterize the community make-up of samples at the domain level. After normalization with reference organisms for Bacteria

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emi_575.fm Page 6 Friday, February 13, 2004 4:23 PM

(*E. coli* B13711) and Archaea (*M. acetivorans* C2A), approximate percentages of domain-specific 16S rRNA were calculated for each rumen sample. Bacterial 16S rRNA comprised approximately 55.5% and 57.4% of the total 16S rRNAs in samples #218 and #256, respectively. The Archaeal contributions to the total 16S rRNA were approximately 25.3% for sample #218 and 24.4% for sample #256.

Discussion

The application of high throughput sequencing technologies to the genomics of environmental microbial communities or metagenomes is providing a wealth of genomic information concerning the ecology, structure and function of the microbial assemblages in these environmental niches (DeLong, 2002). These strategies for analysing the entire metagenome have incorporated nucleic acid based cloning and sequencing approaches which have to date centred on either SSU rRNA genes (Olsen *et al*., 1986), or the generation of large DNA fragment libraries using lambda or BAC vectors (Béjà *et al*., 2000; Rondon *et al*., 2000; DeLong, 2002). As far as we know, the rapid and cost effective methods of SSH or representational difference analysis (Lisitsyn *et al*., 1993) have not been applied to metagenomic analysis of environmental microbial communities. We have used SSH in a pilot study to identify unique DNA sequences found in the metagenome of one rumen sample but absent from another rumen sample, thus demonstrating the utility of this technique for genomic analysis of the functional and structural diversity of complex microbial assemblages in the environment. It should be noted that in order to obtain a comprehensive appraisal of the genetic diversity existing between such complex metagenomes as rumen communities, hundreds of thousands of subtractive fragments must be sequenced. In addition, subtraction fragment redundancy, as observed in this study, may be a limitation in the utility of SSH for comprehensive analyses of genetic diversity between environmental samples. Further experimentation is needed to determine the sources of this redundancy. However, the intent of this study was not to uncover every genetic difference between these metagenomes, but to determine if SSH retains its capability to isolate DNA unique to one sample and absent from another in such a challenging environment.

We have demonstrated that SSH is an effective method to isolate unique DNA fragments present in rumen metagenomic samples. Subtractive hybridization fragments represented cellular functions ranging from energy production and amino acid metabolism to DNA replication, recombination, repair, transcription, and translation, with no apparent pattern or trend. Our finding that almost half of our isolated DNA fragments had no known matches in existing databases was somewhat expected, because of the technique's ability to enrich for unique DNA fragments. This result is also consistent with the nature and complexity of the samples used. Because the rumen ecosystem provides an incredibly diverse population of microorganisms, it is likely that some of these have yet to be identified or sequenced. As databases grow, it is probable that many of the unknown DNA sequences can be identified. Additionally, performing a subtractive hybridization experiment using a restriction endonuclease with a six bp recognition sequence may lead to longer subtracted fragments and a greater probability of finding high similarity matches.

Subtractive hybridization fragments of both Bacterial and Archaeal origin were identified which confirm this technique's ability to provide supplementary sequence information to data generated by other methods for metagenomic analysis of complex microbial environments. However, the distribution of subtractive hybridization fragments with significant database similarities among different domains of life was surprising (Table 1 and Fig. 2). These results may reflect interesting and important differences in the microbial populations of rumen samples from two animals fed an identical diet. Structural differences in the Bacterial populations of the rumen samples were expected given the great diversity of Bacterial species present within the rumen. However, even though all were hypothetical proteins, the significant proportion of similarities to *C. thermocellum* proteins is noteworthy and possibly indicates diversity in cellulolytic organisms between the rumen samples.

Conversely, the high proportion (15/81 or 18.5%) of the total similarity matches to sequences from Archaea was unexpected, as estimations of the rumen Archaeal populations range from 0.5 to 3% of the total microbial population (Lin *et al*., 1997; Ziemer *et al*., 2000). *Methanobrevibacter ruminantium*, *Methanobacterium formicicum*, *Methanomicrobium mobile* and *Methanosarcina barkeri* are the most commonly isolated ruminal Archaea (Hespell *et al*., 1996). The majority of subtractive hybridization fragments showing similarity to Archaeal sequences matched with database entries from *Methanothermobacter thermautotrophicus*, a genus not found in the rumen. Only three of the subtractive hybridization fragments resembled database entries from Archaea found in the rumen. This discrepancy is likely a function of the composition of currently available sequence databases. As databases expand, we would expect to see a shift in our data from the protein similarities reported in this work.

To further support our finding of a high proportion of Archaeal sequences recovered from our subtraction study, we performed quantitative dot blots of rRNA from rumen samples. Examination of rumen community

emi_575.fm Page 7 Friday, February 13, 2004 4:23 PM

domain structure by 16S rRNA hybridizations revealed similar percentages of Bacteria and Archaea in samples #218 and #256. Bacteria contributed over half of the total 16S rRNAs in both rumen sample #218 (55.5%) and sample #256 (57.4%). In addition, high percentages of Archaeal 16S rRNA were found in samples #218 (25.3%) and #256 (24.3%), which was surprising in consideration of previous estimates (Lin *et al*., 1997; Ziemer *et al*., 2000). The large percentage of rumen Archaea displayed by 16S rRNA hybridization methods provides greater evidence that the high proportion of Archaeal sequence similarities in the SSH data is not artifactual. The similar amounts of domain-specific 16S rRNA also suggest that differences in rumen community structure revealed by SSH are due almost entirely to species variation between samples, and are not merely a result of highly unbalanced domain compositions between these communities. That is to say, for example, the high proportion of Archaeal-like sequences uncovered by SSH are not the result of one community containing 25% Archaea and the other community containing 1% Archaea.

Nonetheless, the high proportion of similarity matches to sequences from the Archaea, demonstrate two important aspects of the application of SSH to environmental metagenomes. First, this is the initial report of SSH for analysis of genomic differences in Archaea, even though this method has been applied to the analysis of *T. maritima* (Nesbø *et al*., 2002) a member of the Bacterial domain whose genome may be nearly one quarter Archaeal in nature (Nelson *et al*., 1999). Second, this metagenomic approach has revealed a surprisingly large difference in Archaeal community structure between the rumen microbial assemblages of two steers fed identical diets and housed together. Further experimentation is needed to examine the extent and nature of Archaeal population disparities between rumen samples of animals fed different diets. Therefore, the SSH approach represents an efficient methodology for identifying unique genes present in one complex microbial community and absent from another, and will likely provide a valuable complementary approach to current molecular methods for the analysis of various microbial environments. Further research on the genetic diversity uncovered by SSH should provide insights into relationships between microbial ecosystem structure, community function and microbial interactions, and may determine whether this diversity is representative of major population differences.

Experimental procedures

Rumen sampling and total DNA extraction

Representative samples of rumen contents were taken from two cannulated steers maintained at the University of Illinois at Urbana-Champaign Research Farm (IL, USA) (Kocherginskaya *et al*., 2001). Samples were collected and processed as reported by Peter *et al*. (2000) and Kocherginskaya *et al*. (2001), and stored at −20°C until DNA extraction was performed. Extraction of total genomic DNA from rumen samples was performed using the method of Tsai and Olson (1991) with addition of 50 μg ml^{-1} of proteinase K to samples following the freeze-thaw cycles.

Suppressive subtractive hybridization

Suppressive subtractive hybridization was used to isolate DNA fragments present in the microbial metagenome of one rumen community DNA sample, but absent from the metagenome of the other sample. The procedure was performed using the PCR-Select Bacterial Genome Subtraction Kit (Clontech) with minor modifications. Rumen samples were named using the number of the animal from which they were collected (#218 and #256), and were arbitrarily assigned as tester (#256) or driver (#218). Tester and driver samples were digested separately with *Alu* I.

Polymerase chain reaction amplification of tester-specific fragments was performed using primers directed at tester-ligated adaptor sequences. The TaKaRa *ExTaq* PCR kit (TaKaRa) and a GeneAmp PCR System 2400 thermal cycler (Perkin Elmer) were used for both primary and secondary PCR amplifications. Both PCR amplifications were performed using primers and recommended concentrations of primer and template from the Clontech kit plus 1 × *ExTaq* reaction buffer, 10 mM of each deoxynucleotide triphosphate, and 1.25 U of *ExTaq* DNA polymerase adjusted to a total volume of 25 μl. Primary PCR cycling conditions were: one incubation of 94°C for 5 min followed by 30 cycles of 94°C for 10 s, 66°C for 30 s, and 72°C for 1.5 min, and one final incubation of 72°C for 7 min. Secondary PCR cycling involved a similar program, but used an annealing temperature of 68°C and only 12 cycles.

Four μl of secondary PCR products were cloned into the pCR4-TOPO (Invitrogen) vector and transformed into *E. coli* TOP10 cells (Invitrogen) by electroporation. Ninety-six random transformants were picked and grown at 37°C in 200 μl of Luria–Bertani medium with ampicillin for 2 h. One μl of each cell culture was subjected to PCR using the cycling conditions from primary PCR, but the primers from secondary PCR to verify the presence of clone inserts and for use in dot and Southern blot hybridizations. Transformant stock cultures were maintained at −80°C in Luria–Bertani media with glycerol.

Dot blot and Southern blot hybridization

Dot and Southern blots were prepared using standard procedures (Ausubel *et al*., 2001). For dot blots, PCR products from each of the 96 randomly selected transformants were purified using the Qiaquick PCR Purification Kit (Qiagen) and 10 μl of PCR products were diluted with 40 μl of 1 M NaOH, 5 μl of 200 mM EDTA, and 45 μl of sterile water. Diluted PCR products were denatured, and spotted directly onto Hybond-N + nylon membranes (Amersham) using a 96-well manifold (Gibco BRL). Membranes were UV cross-linked using

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emi_575.fm Page 8 Friday, February 13, 2004 4:23 PM

Stratalinker (Stratagene) and stored dry before hybridization. Membranes were prehybridized for 15 min in a hybridization oven with 7 ml of warm (68°C) Perfect Hyb Plus (Sigma). Probes were made with 100 ng of purified, *Alu* I digested, driver or tester DNA and labeled with [^{32}P-α]-dCTP using the Random Primers DNA Labeling System (Invitrogen) for 16 h, at 68°C in a rotating hybridization oven. All probes had specific activity measurements between 10^7 and 10^9 cpm μg^{-1}. Standard protocols for membrane washing (Ausubel *et al*., 2001) were followed, washing twice under low stringency conditions (50 ml of 2 × SSC + 0.1% SDS, 10 min, room temperature), and twice under moderate stringency conditions (50 ml of 0.2 × SSC + 0.1% SDS, 15 min, 42°C). Membranes were exposed to phosphorimaging plates for 20 min, and the BAS-1800 II system (Fujifilm) was used to capture images.

For each Southern blot, approximately 50 ng of a unique subtracted fragment, 1 μg of tester *Alu* I digest, and 1 μg of driver *Alu* I digest were loaded onto 1.0% agarose gels and electrophoresed for 3 h at 80 V. DNA was transferred from gels to Hybond-N+ nylon membranes (Amersham) overnight, following standard procedures (Ausubel *et al*., 2001). Membranes were rinsed in 2 × SSC and UV cross-linked. Hybridization and imaging procedures were the same as dot blot methods.

Sequencing and data analysis

Sequencing of subtracted inserts was performed using Big Dye terminator chemistry by automated methods at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. Sequence editing, alignment and homology searches were completed by the Bioinformatics Unit at the W.M. Keck Center, University of Illinois at Urbana-Champaign. Sequence data for all 96 clones were submitted to GenBank under the accession numbers CC799160-CC799240.

Total RNA extraction and 16S rRNA dot blot hybridization

Total RNA was extracted from rumen samples #218 and #256, and pure cultures of reference organisms, *E. coli* BW13711 and *Methanosarcina acetivorans* C2A, using slightly modified methods of Lin *et al*. (1997) and Stahl *et al*. (1988). Before processing, one gram samples of rumen fluid and the pellets from 40 ml cultures of *E. coli* BW13711 or *M. acetivorans* C2A were each combined with 400 μl of 0.1 M sodium phosphate buffer pH 6.0, 400 μl of acetate buffer pH 5.1 (50 mM sodium acetate, 10 mM EDTA), 100 μl of 20% SDS (w/v), 500 μl of phenol (buffered to pH 5.1), and 0.1 g of autoclaved zirconium beads (0.1 mm diameter). Cells were lysed by mechanical disruption using a mini-beadbeater reciprocating shaker (Biospec Products, Bartlesville, OK). Samples were homogenized in the beadbeater followed by a 2 minute incubation in a 60°C water bath. The homogenizing and heating steps were repeated twice more. Then, samples were centrifuged at 4°C using a tabletop centrifuge at 10 000 *g* for 5 min. Supernatants were transferred to clean tubes and extracted once with phenol (buffered at 5.1), twice with phenol:chloroform:isoamyl alcohol (125 : 24 : 1; v:v:v), and once with chloroform:isoamyl alcohol (24 : 1; v:v). Total nucleic acids were precipitated from samples by addition of 50 μl of sodium acetate (3M) and one ml of cold absolute ethanol to the supernatant followed by chilling at −80°C for 30 min. Samples were centrifuged at 4°C for 15 min at 10 000 *g* to pellet nucleic acids. The pellets were washed with 100 μl of 80% ethanol (v/v), dried, and resuspended in 100 μl of RNAse-free water. The quantity and integrity of recovered RNA was checked for degradation and contaminating genomic DNA by agarose gel electrophoresis (1% TBE) and spectrophotometry. Samples were treated with 1 U μg^{-1} RQ1 RNAse-free DNAse (Promega) for 30 min at 37°C followed by another extraction with phenol, phenol:chloroform:isoamyl alcohol, and chloroform:isoamyl alcohol performed in the same manner as the above described extraction. Nucleic acids were precipitated, pelleted, and washed as described above and resuspended in 40 μl of RNAse-free water. Samples were electrophoresed (1% TBE) and measured by spectrophotometry once more to assess quantity and confirm the absence of DNA. Each sample of RNA was adjusted to a concentration of 100 ng μl^{-1}.

RNA from rumen samples #218 and #256, *E. coli* BW13711, and *Methanosarcina acetivorans* C2A were denatured and spotted directly onto nylon membranes and cross-linked using the method for dot blotting described above. Each RNA sample was spotted in triplicate at amounts of 100 ng, 50 ng, 20 ng, 5 ng, 2 ng, 0.5 ng and 0.2 ng of RNA per spot (each spotted in a volume of 100 μl). Membranes were prehybridized for 2 h in a hybridization oven with 7 ml of warm (40°C) Perfect Hyb Plus (Sigma). Three DNA oligonucleotide probes were used to target either universal (S-*-University-1392-a-A-15, Olsen *et al.* 1986) or domain-specific (S-D-Bact-0338-a-A-18, Stahl and Amann, 1991 and S-D-Arch-0915-a-A-20, Raskin *et al.* 1994) regions of 16S rRNA. A 10-fold excess of the amount of probe necessary to hybridize to the 16S rRNA on the membranes was end-labelled with ^{32}P using polynucleotide kinase (Invitrogen) and [γ-^{32}P] ATP by the method of Raskin *et al.* (1994) and hybridized to membranes for 16 h, at 40°C in a rotating hybridization oven. All probes had specific activity measurements between 10^7 and 10^9 cpm μg^{-1}. Membranes were washed twice in 1 × SSC + 1% SDS at 40°C for one hour and once at wash temperatures specific for each probe (S-*-University-1392-a-A-15, 46°C; S-D-Bact-0338-a-A-18, 48°C; S-D-Arch-0915-a-A-20, 56°C) for 30 min. Membranes were exposed to phosphorimaging plates for 15 min, and images were captured as described above. ImageQuant software (Fujifilm) was used for densitometry measurements on each dot blot.

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emi_575.fm Page 9 Friday, February 13, 2004 4:23 PM

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emi_575.fm Page 10 Friday, February 13, 2004 4:23 PM

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