'Unculturable' bacterial diversity: An untapped resource

Rakesh Sharma[1,2,*], Ravi Ranjan[2], Raj Kishor Kapardar[1] and Amit Grover[2]

[1]Environmental Biotechnology Unit, Institute of Genomics and Integrative Biology, Mall Road, New Delhi 110 007, India
[2]Department of Environmental Biology, University of Delhi, Delhi 110 007, India

More than 99% of bacteria from environmental samples remain 'unculturable' in the laboratory. Many of these 'unculturable' bacteria represent new phylotypes, families and divisions in domain bacteria and archaea. 'Unculturable' bacterial diversity presents a vast gene pool for biotechnological exploitation and poses a major challenge for microbiologists to understand their phylogenetic relationship and ecological significance. New culture techniques along with culture-independent methods, like PCR amplification from microbial community DNA (metagenome) and functional or sequence-based screening of metagenomic DNA libraries are proving useful for exploitation of 'unculturable' bacteria. Environmental genome sequencing efforts are aiding better understanding of the community structure and their physiological importance in the ecosystem.

HUMAN pursuit has led to cultivation of bacteria from almost all possible habitats on earth for studies related to bacterial diversity, diseases, ecological functions and biotechnological applications. Bacteria were initially isolated from habitats commonly associated with humans, at near neutral pH and ambient temperature. Later these were isolated from even the most hostile environments, like thermal vents, acidic ponds, saturated brine and glaciers[1]. Successful isolation of bacteria from earth's crust[2] and polar ice[3] has led to the belief that bacterial life may exist even on other planets. Though the search to find bacterial life on other planets is ongoing[3], we are still far away from understanding the bacterial diversity in even the most common and well-studied niches[4,5].

Bacterial diversity, the 'unculturables'

Studies in the last two decades have revealed that most (more than 99%) of the bacteria present in many environmental samples cannot be cultivated in the laboratory and hence remain obscure for their ecological functions, and unexploited for biotechnological applications[6]. Culture-independent studies (mostly based on 16s rRNA gene sequence analysis) have made it apparent that a large proportion of these 'yet to be cultivated' bacteria belong to new genotypes, classes and divisions in the domains eubacteria and archaea[5,7]. These studies have revealed presence of newer bacteria even in samples thought to be most well characterized like dental plaques[4], sea water[8] and garden soil[5]. Molecular analysis of bacterial diversity has already resulted in identification of 40 divisions in eubacteria, including 13 candidate divisions, which are not represented by any cultured member[9]. These studies have also revealed presence of archaea in habitats like soil[5], seawater[10], etc., contrary to previous belief that archaea inhabited only the extreme environments.

Cultivating the 'unculturables'

Abundance and diversity of unculturable bacteria in almost all environmental niches have led to the understanding that the so-called 'unculturable' bacteria actually multiply in their natural environment and if suitable culture conditions were provided it should be possible to cultivate them in the laboratory. In the earliest cultivation attempts, media with very low nutrient were used considering the high nutrient contents of common laboratory media as compared to those present in the natural environments[11–13]. Successful isolation of many new genera by these methods including members of the candidatus clade[14] like SAR11 resulted in more efforts in this direction. Recently, using simple media and physiological conditions, many previously unknown bacteria have been isolated which belong to diverse families and phyla[15,16]. Extinction culturing technique with low nutrient media was used to culture previously uncultured marine bacteria belonging to SAR11, OM43, SAR92 and OM60/OM241 clades of proteobacteria[13]. A collection of 350 isolates from soil was obtained using a variety of simple solid media in petri dishes. Twenty seven per cent of these isolates belonged to 20 as yet unnamed family level groupings. Many of these isolates were members of poorly studied subdivisions of phyla *Acidobacteria*, *Verrucomicrobia, Gemmatimonadetes* and *Actinobacteria*[15]. Recently, bacteria from soil and termite gut were isolated by an integrated approach using various growth parameters like low nutrient media, varying oxygen and carbon dioxide concentrations, long incubation period and additives like humic acids and quorum signalling molecules. It was observed that more isolates belonging to *Acidobacteria* were obtained in presence of higher concentration of carbon dioxide[16].

*For correspondence. (e-mail: rsharma@igib.res.in)

Other innovative and successful approaches considered include, simulation of natural environment, community interactions and cell–cell communication important for the cultivation. Kaeberlein *et al.*[17] designed a diffusion chamber that allowed cultivation of previously uncultivated bacteria in a simulated natural environment. The isolates did not grow on artificial media alone but formed colonies in presence of other microorganisms indicating that they required specific signals originating from their neighbours that point to the presence of familiar environment. In another method, single cells were encapsulated in gel microdroplets and allowed to grow with nutrients that were present at environmental concentrations in a single vessel. The pore size in gel microdroplets allowed exchange of metabolites and signalling molecules, which might be the reason for the enhanced culturability[18].

Mining the 'unculturables'

Culture-independent molecular microbial diversity studies not only revealed the vast unexplored bacterial diversity but also helped in development of techniques suitable for isolation of high purity microbial community DNA (metagenome) from various environmental samples. The metagenomic DNA gave molecular biologists a chance to peek into the genomes of 'unculturable' bacteria, circumventing the need to culture them in the laboratory. The possibility of getting access to the huge and diverse gene pool of 'unculturable' bacteria led to a 'gold rush' in the last decade, with publications describing innovative techniques to exploit metagenomes for novel biocatalysts and bioactive compounds (Table 1). The metagenomes from many habitats have been exploited for isolation of novel genes by PCR amplification using primers against the conserved domains of known genes or by preparation of metagenomic libraries containing small inserts of 2–15 kb in plasmid vectors or large inserts of 40–130 kb in cosmid, fosmid or bacterial artificial chromosome (BAC) vectors (Figure 1).

PCR cloning

Two β-ketoacyl genes, components of bacterial type II polyketide synthases, were cloned from environmental DNA by PCR amplification using primers against the conserved domain of known ketosynthase and acyl carrier protein (ACP) genes[19]. PCR amplification using primers against conserved domains and genome walking resulted in isolation of two complete genes for 2,5-diketo-D-gluconic acid reductase. Compared to previously known 2,5-diketo-D-gluconic acid reductases, they had some valuable properties like lower K_m values and higher thermostability[20]. PCR method was also used to explore the diversity of chitinase genes in culturable and unculturable marine bacteria[21]. A lipase gene with less than 20% similarity to known lipases at amino acid level was cloned from soil DNA using primers against the conserved domains of the known lipases and genome walking to obtain the full length gene[22]. A major disadvantage of PCR-based method is that the sequence information of known genes from culturable bacteria is used, thus eluding the possibility of finding completely novel genes. Gene cassette PCR method was devised using 59 base element family of recombination sites as target. These sites flank gene cassettes associated with integrons. This method allowed sequence-independent recovery of entire genes from environmental DNA. Genes with different possible functions were amplified using this method from soil metagenome[23].

Table 1. Biocatalysts and bioactive compounds isolated from metagenomic libraries

Biocatalyst and bioactive compound	Vector	Environmental sample	Reference
Agarase	Cosmid	Soil	43
Alcohol oxidoreductase	Plasmid	Soil, sediment	28
Amidase	Cosmid	Soil	43
Amylase	Cosmid, BAC	Various samples, soil	27, 36, 43
Cellulase	Cosmid	Soil	43
Chitinase	Lambda-ZAP	Marine water	24
DNase	BAC	Soil	36
Esterase	Plasmid	Soil	26
Glycerol/diol dehydratase	Plasmid	Soil, sediment	32
4-Hydroxybutyrate dehydrogenase	Plasmid	Soil	25
Lipase	Plasmid, cosmid, BAC	Soil	22, 26, 36, 43
Nitrilase	Lambda-ZAP	Various samples	29
Pectate lyase	Cosmid	Soil	43
Xylanase	Lambda-ZAP	Insects gut	30
Indirubin	BAC	Soil	40
Long chain *N*-acyl aromatic amino acids	Cosmid	Soil	38
Polyketide synthase	Cosmid	Soil	42
Turbomycin	BAC	Soil	41
Violacein, deoxyviolacein	Cosmid	Soil	38

Plasmid cloning

Small insert metagenomic libraries were prepared using *Escherichia coli* as host for various environmental samples including marine water, sediments and soil from various regions. These libraries were used for functional screening of clones expressing various enzymes like chitinase[24], hydroxybutyrate dehydrogenase[25], lipase and esterase[26], amylase[27], alcohol oxidoreductase[28], nitrilase[29] and xylanase[30]. Two libraries prepared from coastal and estuarine waters were screened for clones expressing chitinase. Nine clones were obtained from 75,000 colonies screened from estuarine sample and one clone for every 500 recombinants was tested from coastal library. The results were consistent with the culture-based estimates of the proportion of marine bacteria that degrade chitin[24]. Three different soil libraries were constructed and screened for various enzymatic activities. These libraries containing more than 930,000 recombinants were screened to obtain five clones that grow with 4-hydroxybutyrate as sole carbon and energy source. Gene sequences from three clones showed similarity to known genes of 4-hydroxybutyrate dehydrogenase, member of *Ded*A family and enoyl coenzymeA hydratase/isomerase. The other two sequenced inserts showed no homology to known genes in databases[25]. One lipase and three esterase clones were obtained by screening the three soil libraries. The deduced protein sequences of these four clones showed less than 50% amino acid identity to known sequences in databases, indicating that sequence information collected solely with cultivated microorganisms was not sufficient to design universal primers to retrieve the variety of genes encoding lipolytic enzymes from natural microbial communities[26]. Utilizing the same three soil libraries, Majernik *et al.*[31] complemented the Na^+/H^+ antiporter deficient *E. coli* strain. They obtained two clones, one containing a novel Na^+/H^+ antiporter belonging to NhaA family and the other with the DNA region of *E. coli* K12 containing *nhaA, nhaR* and *gef*. A novel amylase with an exceptional process compatibility and economics was obtained by a combination of environmental library screening and laboratory evolution approaches[27]. Screening of >600-biotope specific environmental DNA libraries containing 10^6 to 10^{10} members per library by selection-based expression assay resulted in isolation of 137 nitrilases. Sequence analysis revealed five major sequence clades within the nitrilase subfamily[29]. Libraries prepared from the intestinal tracts of termites and moths were screened to obtain unusual microbial xylanases. Biochemical analysis revealed that they were true xylanases though their sequences were remarkably different from the known xylanases[30].

In another technique which combined traditional enrichment culture and metagenomic approach, a gene bank of alcohol oxidoreductases was generated containing 24 clones. These clones were completely sequenced. Sixteen of them conferred carbonyl forming phenotype and eight clones exhibited NAD(H)-dependent alcohol oxidoreductase activity[28]. Libraries from three different environmental samples were prepared after enrichment culture with glycerol under anaerobic conditions and screened by functional assay and colony hybridization to obtain glycerol dehydratase and diol dehydratase encoding genes. Out of the seven clones obtained, two clones were similar to glycerol dehydratase of *Citrobacter freundii* and the other five contained dehydratase encoding gene region similar to enteric bacteria[32].

Cosmid, fosmid or bacterial artificial chromosome cloning

Large insert libraries are particularly suitable for the screening of operons and pathways for the novel bioactive compounds. Cosmid or fosmid libraries containing inserts of about 40 kb and bacterial artificial chromosome (BAC) libraries containing inserts up to 150 kb have been prepared from metagenomic DNA. The major difficulty in preparation of large insert libraries is isolation of high molecular weight metagenomic DNA, which is a challenging task for samples rich in organic components, humic

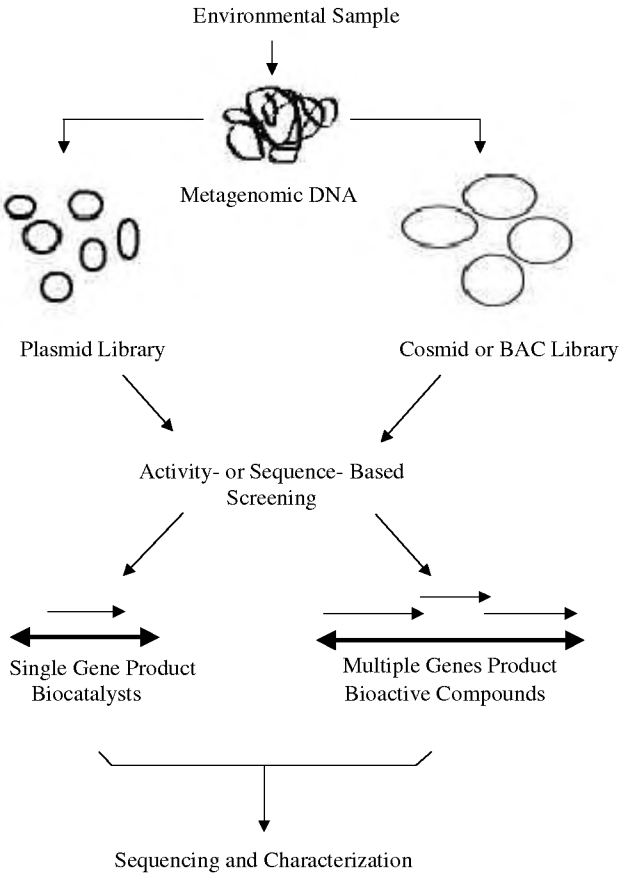

Figure 1. Culture-independent approach to assess the 'unculturable' bacterial diversity for novel biocatalysts and bioactive compounds.

acids and heavy metals[33]. This could be the reason for early success in preparation of large insert metagenomic libraries from marine samples. A fosmid library containing inserts of ~40 kb marine bacterial DNA was constructed by Stein *et al.*[34] followed by preparation of BAC library of marine microbial assemblages containing inserts up to 150 kb with an average insert size of 80 kb[35]. Rondon *et al.*[36] were the first to prepare large insert BAC libraries from soil metagenomic DNA for screening of antibiotics and biocatalytic activities. Two metagenomic BAC libraries, having average inserts size of 27 kb and 44.5 kb, containing more than 1 Gbp of soil DNA were prepared. Phylogenetic analysis of 16S rRNA genes amplified from these libraries indicated presence of DNA of wide diversity of microbial phyla. Active clones with antibacterial, lipase, amylase, nuclease and hemolytic activity were identified from these two libraries. Isolation of long chain acyltyrosine antibiotics, derived from a single environmental DNA open reading frame, were reported in another study[37], followed by identification of a biosynthetic gene cluster that produced two additional families of natural products derived from long chain *N*-acyltyrosines[38].

A blue clone was isolated and characterized from environmental DNA cosmid library[39]. Colour-producing clones often indicate presence of small molecules. Characterization of blue clone revealed presence of a four-gene biosynthetic cluster conferring production of violacein and deoxyviolacein in *E. coli* host. The DNA sequence of the gene cluster resembled with that of violacein gene cluster sequenced from cultured bacterium *Chromobacterium violaceum*. A BAC soil DNA library, containing inserts between 5 and 120 kb resulted in isolation of many antibacterial clones. One of these clones contained indirubin and related small molecules[40]. In another study, characterization of dark brown colour producing BAC metagenomic clones revealed presence of broad spectrum antibiotic compounds Turbomycin A and Turbomycin B. Sequence analysis of the clones revealed that a single open reading frame was necessary and sufficient to confer brown, orange and red pigments on *E. coli*. It was demonstrated that interaction of indole with homogentistic acid, synthesized by 4-hydroxyphenylpyruvate dioxygenase encoded by open reading frame from the BAC, resulted in production of Turbomycin A and Turbomycin B in recombinant clone[41].

A large number of antibiotics and bioactive compounds are synthesized by actinomycetes in nature. It was realized that it would be advantageous to prepare metagenomic libraries in *Streptomyces* as it will help in better expression of the genes from related unculturable actinomycetes. A cosmid library prepared in shuttle cosmid vector was transformed in *Streptomyces lividans* and screened for polyketide synthase genes by PCR and presence of novel molecules by high performance liquid chromatography. Eight new polyketide synthase genes and five clones producing new molecules were identified[42]. Voget *et al.*[43] utilized the precultivation-metagenomic technology for preparation of cosmid library from a microbial consortium. This library was successfully used for prospecting of various biocatalysts like agarase, amidase, amylase, cellulase, pectate lyase and lipase[43]. In an attempt to utilize the expression capabilities of different hosts like *E. coli*, *Streptomyces lividans* and *Pseudomonas putida* new tools were developed. These tools included a new BAC vector capable of transferring large fragments of environmental DNA among these three hosts, modified *S. lividans* and *P. putida* strains and high throughput BAC library transfer methods[44]. The development of new tools exploiting expression capabilities of multiple hosts, libraries prepared from metagenomic DNA from various habitats, in combination with high throughput screening is expected to yield novel and potent bioactive compounds in near future.

Understanding the 'unculturables'

One to two orders of magnitude higher populations count by microscopic observation than that measured by conventional culturing of the same sample[45] indicated presence of a large number of 'unculturable' bacteria in the environment. Microbial diversity analysis by 16S rRNA gene sequences confirmed their presence and diverse phylogenetic linkages but this method failed to elucidate their physiology, abundance and ecological significance[46]. Attempts in this direction would help in understanding ecosystem function, community structure and also in designing newer techniques to cultivate the 'unculturable' bacteria. Genomic information of the 'unculturable' bacteria can be obtained from the clones in metagenomic libraries, which can help in understanding their physiology and also about their role in ecosystem. Some success has been obtained in achieving these goals in recent years. Sequence analysis of a 130 kb genomic fragment from a BAC clone that encoded the rRNA operon from an uncultivated member of marine γ-proteobacteria (SAR 86 group) revealed the presence of rhodopsin gene. This gene encoded a protein with highest amino acid sequence similarity with archaeal rhodopsins. The halophilic archaea contain bacteriorhodopsins that function as light-driven proton pump for energy generation. This first report of the presence of rhodopsin in marine γ-proteobacteria in domain bacteria suggested the possibility of a previously unrecognized phototrophic pathway that may influence the flux of carbon and energy in the ocean's photozone worldwide[47].

The contents and structural comparison of 34 kb fragment containing 16S/23S rRNA operon and 17 genes encoding putative proteins from a non-thermophilic clade of soil crenarchaeota (archaea) revealed significant differences from their previously studied marine relative[48]. In order to understand the physiology of the yet to be cultured members of the *Acidobacteria* division, a BAC clone containing full rRNA gene and 20 other open reading

frames was completely sequenced[49]. Genes for cell division, cell cycling, folic acid biosynthesis, substrate metabolism, amino acid uptake, DNA repair and transcriptional regulation were identified. Advancement in sequencing technology, reduction in sequencing cost and higher computational capabilities have made it possible to sequence complete metagenomes using shotgun metagenomic libraries and assembly of near complete individual genomes from mixed sequences[50,51]. These efforts are a major advancement from the limited genes identification for biotechnological applications to comprehensive genomic data, which would resolve organism-specific pathways and provide insight into population structure, speciation and evolution[51]. Two almost complete genomes of *Leptospirillum* group II and *Ferroplasma* type II, and partial recovery of three other genomes were obtained by shotgun sequencing of DNA from a natural acidophillic biofilm. Sequence analysis revealed pathways of carbon and nitrogen fixation and energy generation for each organism of the simple biofilm community. It also suggested role of *Leptospirillum* group II in biofilm formation and possibility of *Leptospirillum* group II and III as first colonizers of the biofilm[50]. Venter *et al.*[51] sequenced environmental genome of the Sargasso Sea in an attempt to analyse gene contents, diversity and relative abundance of the organisms in the sample. The data obtained by this major sequencing effort led to identification of more than 1.2 million previously unknown genes and assembly of groups of scaffolds closely related to *Burkholderia, Shewanella oneidensis*, SAR 86 and a conglomerate of *Prochlorococcus* strains. Phylogenetic analysis of the sequence of 16S rRNA genes revealed presence of 148 previously unknown phylotypes based on 97% similarity cut-off to distinguish unique phylotypes. An ammonium monooxygenase gene was found on an archaeal-associated scaffold indicating their role in oceanic nitrification. Only members of bacterial domain were known for ammonium monooxygenase prior to this study. The presence of genes for transport of phosphonates, utilization of polyphosphates and pyrophosphates, phosphorus transporter *pst*S and *pho* regulon group of genes indicated capability of the Sargasso Sea's microbial community to survive in an extremely phosphate-limited environment[51].

Conclusions

Culture-independent microbial diversity analysis in the last decade has revealed previously uncharacterized members in both bacterial and archaeal domains. These novel 'yet to be cultured' bacteria represent an unexplored and unexploited vast gene pool. Attempts to culture these unique bacteria or screening of metagenomic libraries, circumventing the need of culture, have found an impetus in recent times. Development of new culture techniques and more innovative tools in molecular biology related to genomic library construction in culturable members of various bacterial groups are expected to revolutionize the field of biocatalysts and drug discovery. Further decrease in sequencing cost is expected to increase efforts for microbial community genome sequencing in an attempt to understand community structure and ecosystem function. Availability of community genome sequences will help in development of gene expression profiles and physiological studies providing a comprehensive approach to environmental biology[51].

1. Madigan, M. T. and Marrs, B. L., Extremophiles. *Sci. Am.*, 1997, **276**, 66–71.
2. Kerr, R. A., Life goes to extremes in the deep earth – and elsewhere? *Science*, 1997, **276**, 703–704.
3. Rothschild, L. J. and Mancinelli, R. L., Life in extreme environments. *Nature*, 2001, **49**, 1092–1101.
4. Kroes, I., Lepp, P. W. and Relman, D. A., Bacterial diversity within the human subgingival crevice. *Proc. Natl. Acad. Sci. USA*, 1999, **96**, 14547–14552.
5. Bintrim, S. B., Donohue, T. J., Handelsman, J., Roberts, G. P. and Goodman, R. M., Molecular phylogeny of archaea from soil. *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 277–282.
6. Kellenberger, E., Exploring the unknown: the silent revolution of microbiology. *EMBO Rep.*, 2001, **2**, 5–7.
7. Hugenholtz, P., Pitulle, C., Hershberger, K. L. and Pace, N. R., Novel division level bacterial diversity in a yellowstone hot spring. *J. Bacteriol.*, 1998, **180**, 366–376.
8. DeLong, E. F., Marine microbial diversity: the tip of the iceberg. *Trends Biotechnol.*, 1997, **15**, 203–207.
9. Hugenholtz, P., Goebel, B. M. and Pace, N. R., Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.*, 1998, **180**, 4765–4774.
10. Furhman, J. A., McCallum, K. and Davis, A. A., Novel major archaebacterial group from marine plankton. *Nature*, 1992, **356**, 148–149.
11. Button, D. K., Schut, F., Quang, P., Martin, R. and Robertson, B. R., Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. *Appl. Environ. Microbiol.*, 1993, **59**, 881–891.
12. Watve, M. *et al.*, The 'K', selected oligophilic bacteria: A key to uncultured diversity? *Curr. Sci.*, 2000, **78**, 1535–1541.
13. Connon, S. A. and Giovannoni, S. J., High-throughput methods for culturing microorganisms in very low nutrient media yield diverse new marine isolates. *Appl. Environ. Microbiol.*, 2002, **68**, 3878–3885.
14. Rappe, M. S., Connon, S. A., Vergin, K. L. and Giovannoni, S. J., Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature*, 2002, **418**, 630–633.
15. Joseph, S. J., Hugenholtz, P., Sangwan, P., Osburne, C. A. and Janssen, P. H., Laboratory cultivation of widespread and previously uncultured soil bacteria. *Appl. Environ. Microbiol.*, 2003, **69**, 7210–7215.
16. Stevenson, B. S., Eichorst, S. A., Wertz, J. T., Schmidt, T. M. and Breznak, J. A., New strategies for cultivation and detection of previously uncultured microbes. *Appl. Environ. Microbiol.*, 2004, **70**, 4748–4755.
17. Kaeberlein, T., Lewis, K. and Epstein, S. S., Isolating 'Uncultivable' microorganisms in pure culture by a simulated natural environment. *Science*, 2002, **296**, 1127–1129.
18. Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E. J., Short, J. M. and Keller, M., Cultivating the uncultured. *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 15681–15686.
19. Seow, K.-T., Meurer, G., Gerlitz, M., Wendt-Pienkowski, E., Hutchinson, R. C. and Davies, J., A study of iterative type II polyketide synthases, using bacterial genes cloned from soil DNA: a means to access and use genes from uncultured microorganisms. *J. Bacteriol.*, 1997, **179**, 7360–7368.

20. Eschenfeldt, W. H. *et al.*, DNA from uncultured organisms as a source of 2,5-diketo-D-gluconic acid reductases. *Appl. Environ. Microbiol.*, 2001, **67**, 4206–4214.
21. Cottrell, M. T., Wood, D. N., Yu, L. and Kirchman, D. L., Selected chitinase genes in cultured and uncultured marine bacteria in the α- and β-subclasses of the proteobacteria. *Appl. Environ. Microbiol.*, 2000, **66**, 1195–1201.
22. Bell, P. J .L., Sunna, A., Gibbs, M. D., Curach, N. C., Nevalainen, H. and Bergquist, P. L., Prospecting for novel lipase genes using PCR. *Microbiology*, 2002, **148**, 2283–2291.
23. Stokes, H. W., Holmes, A. J., Nield, B. S., Holley, M. P., Nevalainen, K. M. H., Mabbutt, B. C. and Gillings, M. R., Gene cassette PCR: Sequence independent recovery of entire genes from environmental DNA. *Appl. Environ. Microbiol.*, 2001, **67**, 5240–5246.
24. Cottrell, M. T., Moore, J. A. and Kirchman, D. L., Chitinases from uncultured marine microorganisms. *Appl. Environ. Microbiol.*, 1999, **65**, 2553–2557.
25. Henne, A., Daniel, R., Schmitz, R. A. and Gottschalk, G., Construction of environmental DNA libraries in *Escherichia coli* and screening for the presence of genes conferring utilization of 4-hydroxybutyrate. *Appl. Environ. Microbiol.*, 1999, **65**, 3901–3907.
26. Henne, A., Schmitz, R. A., Bomeke, M., Gottschalk, G. and Daniel, R., Screening of environmental DNA libraries for the presence of genes conferring lipolytic activity on *Escherichia coli*. *Appl. Environ. Microbiol.*, 2000, **66**, 3113–3116.
27. Richardson, H. T. *et al.*, A novel, high performance enzyme for starch liquefaction. *J. Biol. Chem.*, 2002, **277**, 26501–26507.
28. Knietsch, A., Waschkowitz, T., Bowien, S., Henne, A. and Daniel, R., Construction and screening of metagenomic libraries derived from enrichment cultures: Generation of a gene bank for genes conferring alcohol oxidoreductase activity on *Escherichia coli*. *Appl. Environ. Microbiol.*, 2003, **69**, 1408–1416
29. Robertson, D. E. *et al.*, Exploring nitrilase sequence space for enatioselective catalysis. *Appl. Environ. Microbiol.*, 2004, **70**, 2429–2436.
30. Brennan, Y. *et al.*, Unusual microbial xylanases from insect guts. *Appl. Environ. Microbiol.*, 2004, **70**, 3609–3617.
31. Majernik, A., Gottschalk, G. and Daniel, R., Screening of environmental DNA libraries for the presence of genes conferring $Na^+(Li^+)/H^+$ antiporter activity on *Escherichia coli*: characterization of the recovered genes and the corresponding gene products. *J. Bacteriol.*, 2001, **183**, 6645–6653.
32. Knietsch, A., Bowein, S., Whited, G., Gottschalk, G. and Daniel, R., Identification and characterization of coenzyme B_{12}-dependent glycerol dehydratase- and diol dehydratase-encoding genes from metagenomic DNA libraries derived from enrichment cultures. *Appl. Environ. Microbiol.*, 2003, **69**, 3048–3060.
33. Ogram, A., in *Techniques in Microbial Ecology* (eds Burlage, R. S., Atlas, R., Sthal, D., Geesey, G. and Sayler, G.), Oxford University Press, USA, 1998, pp. 273–288.
34. Stein, J. L., Marsh, T. L., Wu, K. Y., Shizuya, H. and DeLong, F., Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genomic fragment from a marine archaeon. *J. Bacteriol.*, 1996, **178**, 591–599.
35. Beja, O. *et al.*, Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. *Environ. Microbiol.*, 2000, **2**, 516–529.
36. Rondon, M. R. *et al.*, Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.*, 2000, **66**, 2541–2547.
37. Brady, S. F. and Clardy, J., Long-chain *N*-acyl amino acid antibiotics isolated from heterogeneously expressed environmental DNA. *J. Am. Chem. Soc.*, 2000, **122**, 12903–12904.
38. Brady, S. F., Chao, C. J. and Clardy, J., New natural product families from and environmental DNA (eDNA) gene cluster. *J. Am. Chem. Soc.*, 2002, **124**, 9968–9969.
39. Brady, S. F., Chao, C. J., Handelsman, J. and Clardy, J., Cloning and heterologous expression of a natural product biosynthetic gene cluster from eDNA. *Organ. Lett.*, 2001, **3**, 1981–1984.
40. MacNeil, I. A. *et al.*, Expression and isolation of antimicrobial small molecules from soil DNA libraries. *J. Mol. Microbiol. Biotechnol.*, 2001, **3**, 301–308.
41. Gillespie, D. E. *et al.*, Isolation of antibiotics Turbomycins A and B from a metagenomic library of soil microbial DNA. *Appl. Environ. Microbiol.*, 2002, **68**, 4301–4306.
42. Curtois, S. *et al.*, Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. *Appl. Environ. Microbiol.*, 2003, **69**, 49–55.
43. Voget, S., Leggewie, C., Usebeck, A., Raasch, C., Jaeger, K. E. and Streit, W. R., Prospecting for novel biocatalysts in a soil metagenome. *Appl. Environ. Microbiol.*, 2003, **69**, 6235–6242.
44. Martinez, A., Kolvek, S. J., Yip, C. L. T., Hopke, J., Brown, K. A., MacNeil, I. A. and Osburne, M. S., Genetically modified bacterial strains and novel bacterial artificial chromosome shuttle vectors for constructing environmental libraries and detecting heterologous natural products in multiple expression hosts. *Appl. Environ. Microbiol.*, 2004, **70**, 2452–2463.
45. Amann, R. I., Ludwig, W. and Schleifer, K. H., Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.*, 1995, **59**, 143–169.
46. Embley, M. T. and Stackebrandt, E., In *Molecular Approaches to Environmental Microbiology* (eds Pickup, R. W. and Saunders, J. R.), Prentice Hall, UK, 1996, pp. 39–62.
47. Beja, O. *et al.*, Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science*, 2000, **289**, 1902–1906.
48. Quaiser, A. *et al.*, First insight into the genome of an uncultivated crenarchaeote from soil, *Environ. Microbiol.*, 2002, **4**, 603–611.
49. Liles, M. R., Manske, B. F., Bintrim, S. B., Handelsman, J. and Goodman, R. M., A census of rRNA genes and linked genomic sequences within a soil metagenomic library. *Appl. Environ. Microbiol.*, 2003, **69**, 2684–2691.
50. Tyson, G. W. *et al.*, Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature*, 2004, **428**, 37–43.
51. Venter, C. J. *et al.*, Environmental genome shotgun sequencing of the Sargasso sea. *Science*, 2004, **304**, 66–74.

ACKNOWLEDGEMENTS. We thank Prof. S. K. Brahmachari, Director, IGIB for encouragement and support. R.R. and A.G. acknowledge CSIR for junior research fellowships. R.S. thanks DST (FTP/LS-277/2000) and CSIR (SMM0002) for financial support.