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Metagenomics – the key to the uncultured microbes

Wolfgang R Streit*[1,2] and Ruth A Schmitz[1]

It is widely accepted that up to 99.8% of the microbes present in many environments are not readily culturable. 'Metagenome technology' tries to overcome this bottleneck by developing and using culture-independent approaches. From the outset, metagenome-based approaches have led to the accumulation of an increasing number of DNA sequences, but until this time the sequences retrieved have been those of uncultured microbes. These genomic sequences are currently exploited for novel biotechnological and pharmaceutical applications and to increase our knowledge on microbial ecology and physiology of these microbes. Using the metagenome sequences to fully understand how complex microbial communities function and how microbes interact within these niches represents a major challenge for microbiologists today.

Addresses
[1] Institut für Mikrobiologie und Genetik, Universität Göttingen, Grisebachstr. 8, 37077, Göttingen, Germany
[2] Institut für Grenzflächenbiotechnologie, Molekulare Enzymtechnologie, Universtät Duisburg-Essen, Geibelstr. 41, 47057 Duisburg, Germany
*e-mail: wstreit@gwdg.de

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Introduction

Current estimates indicate that more than 99% of the microorganisms present in many natural environments are not readily culturable and therefore not accessible for biotechnology or basic research [1]. In fact, most of the species in many environments have never been described, and this situation will not change until new culture technologies are developed. Additionally, many approaches currently used to explore the diversity and potential of microbial communities are biased because of the limitations of cultivation methods. To overcome the difficulties and limitations associated with cultivation techniques, several DNA-based molecular methods have been developed. In general, methods based on 16S rRNA gene analysis provide extensive information about the taxa and species present in an environment. However, these data usually provide only little if any information about the functional role of the different microbes within the community and the genetic information they contain. By contrast, metagenomics is a new and rapidly developing field, which tries to analyze the complex genomes of microbial niches.

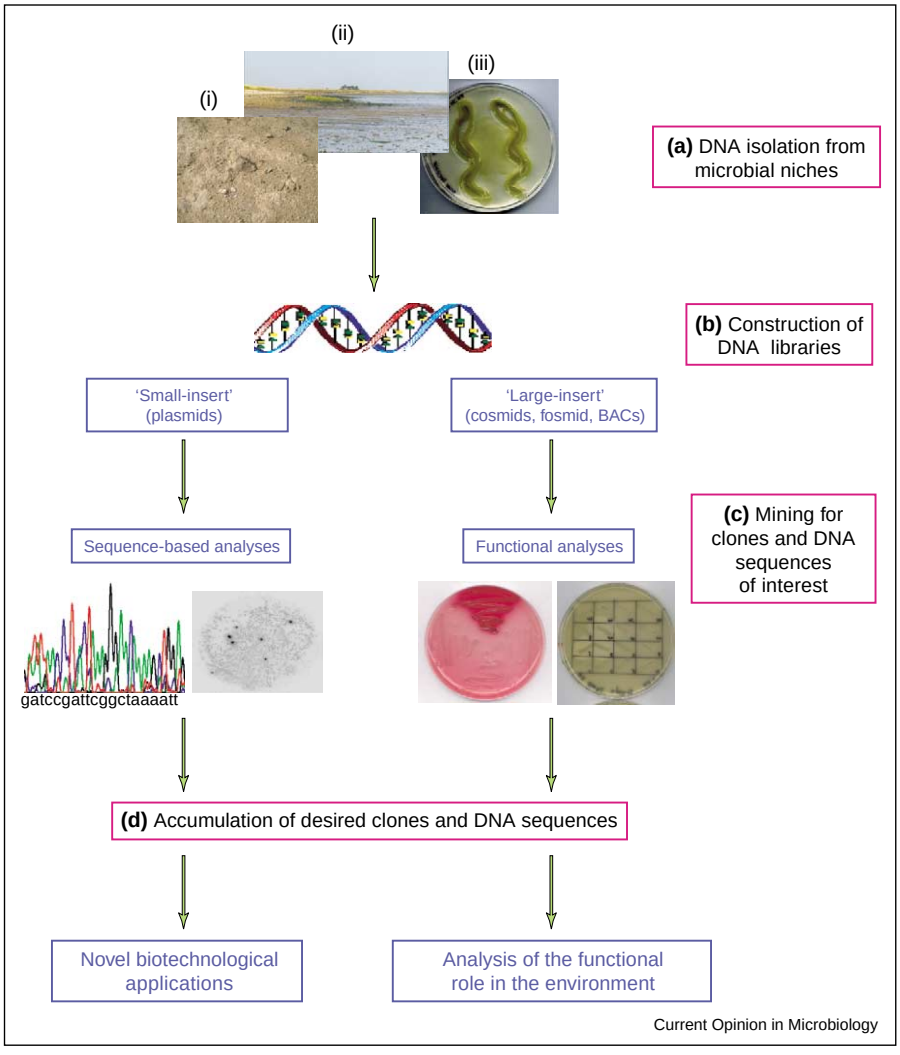
In this review, we discuss the major strategies and working steps used to identify novel genes from uncultured microbes and complex microbial assemblages. Further, we highlight the usefulness of these novel genes for biotechnological or pharmaceutical applications and recent genome observations in metagenomic research. We close with a review on key discoveries from metagenome findings, starting with the initial outline of the concept [2], and their impact on our understanding of fundamental biogeochemical cycles in microbial niches.

Metagenome technologies, DNA extraction, library construction, screening

Metagenome analyses are usually initiated by the isolation of environmental DNAs (Figure 1). A major difficulty associated with the metagenome approach is related to the contamination of purified DNA with polyphenolic compounds, which are copurified with the DNA. These compounds are difficult to remove, and it is well known that polyphenols also interfere with enzymatic modifications of the isolated DNA [3]. In addition, for marine samples often large amounts of water have to be filtered to obtain sufficient DNA for cloning [4]. As a result, the construction of environmentally derived DNA libraries with large inserts is often hindered because of the poor quality of the isolated DNA. These known difficulties associated with the construction of libraries directly derived from environmental DNA samples have forced many laboratories to isolate DNA from the metagenome of a microbial community after pre-cultivation in the laboratory. Although it is expected that laboratory enrichment cultures bear only a limited biodiversity this technique has proven to be highly efficient for the rapid isolation of large DNA fragments and for cloning of operons and genes with high biotechnological value [5•,6–8].

DNA isolation and purification is followed by the construction of DNA libraries in suitable cloning vectors and host strains. The classical approach includes the construction of small insert libraries (< 10 kb) in a standard sequencing vector and in *Escherichia coli* as a host strain [9]. However, small insert libraries do not allow detection of large gene clusters or operons. To circumvent this limitation researchers have been employing large insert libraries, such as cosmid DNA libraries (mostly in the pWE15 vector of Stratagene) with insert sizes ranging

Figure 1



The identification of novel genes and the analysis of complete metagenomes from microbial communities. Analyses of complete metagenomes involves at least four major working steps: **(a)** DNA isolation from microbial niches (i) soils, (ii) sediments, or (iii) laboratory enrichments; **(b)** construction of DNA libraries; **(c)** mining for clones and DNA sequences of interest and; **(d)** accumulation of desired clones and DNA sequences. Metagenomics thereby pursues two major goals: the identification of novel biocatalyst genes and increasing our understanding on microbial ecology.

from 25–35 kb [7] and/or bacterial artificial chromosome (BAC) libraries with insert sizes up to almost 200 kb [4,10]. Additionally, the construction of fosmid libraries with inserts of 40 kb of foreign DNA has been reported [11]. *E. coli* is still the preferred host for the cloning and expression of any metagenome-derived genes. Only very recently have other hosts such as *Streptomyces lividans* been employed to identify genes involved in the biosynthesis of novel antibiotics [12]. Also, to our knowledge metagenomic libraries are currently developed in other Gram-negative hosts by several laboratories working in the field, which will become available soon. The development of these new host strains will increase screening efficiency tremendously.

Functional searches for novel genes in metagenomic libraries have often been performed using highly sophisticated picking and pipetting robots. In many of the recent publications large clone-libraries have been screened. Often several hundred thousand clones have been analyzed to detect less than ten active clones in a single screen [9,13,14]. This is mainly owing to a lack of efficient transcription of the metagenome-derived genes in the host strain. This effect might be worsened by a weak translation in combination with a poor secretion of the foreign protein by the employed host strain. Also, in many cases it can be expected that the desired protein is not folded correctly because required chaperones are absent in the host strain. Furthermore, cofactors might

not be synthesized by the foreign host strain and/or not inserted correctly into the recombinant metagenomic protein. Finally, a different codon usage could be a reason for poor protein expression and low activities. Several laboratories are currently working on solutions for some of these problems and constructing novel vectors and strains. Other avenues to pursue include the development of highly sensitive screening methods using fluorogenic substrates.

Large-scale sequencing projects such as the one initiated by Craig Venter for the metagenome of the Sargasso Sea resulted in the identification of numerous novel genes and is a very famous example of sequence-based metagenome analyses [15•]. Similar approaches have been initiated by European laboratories to sequence complete or partial metagenomes of a phylogenetically highly diverse biofilm [16•]. Complementary to this, Tyson and coworkers described the nearly complete sequence analyses of the metagenome of an acidophilic biofilm, which was characterized by a low biodiversity [17••]. A completely novel strategy has been developed by searching through metagenome DNA sequences and by using microarray technology [18]. The microarray profiling offers an effective approach for rapidly identifying and characterizing many clones. It could potentially be used for the isolation of large numbers of conserved genes. Similarly, other laboratories have been using degenerated PCR primers for the isolation of metagenome-derived genes. Among these are genes involved in the degradation of α-halocarboxylic acids [19] and genes encoding for novel hydrolases [20].

Metagenomics — a key technology for the development of novel biotechnological and pharmaceutical products

Because of the overwhelming majority of non-cultured microbes in most microbial niches, metagenome searches will always result in the identification of hitherto unknown genes and proteins. Thus, the probability of uncovering novel sequences makes this approach more favorable than searches in already cultivated microbes. Since its introduction, metagenomics has identified a significant number of novel genes encoding for biocatalysts or molecules with high potential for use in pharmaceutical products or production processes (Figure 1). Interestingly, searches in metagenome-derived DNA libraries have mainly focused on a rather small group of enzymes. Among these are lipases and esterases [5•,13,16•,20]. Lipases and esterases are of importance for biotechnological applications because many enzymes of this class remain active in organic solvents; they usually display exquisite chemo-, regio-, and stereoslectivities and they do not require cofactors [21–23]. Oxidoreductases are another example of useful catalysts with a high enantioselectivity that have been identified in metagenome searches [6,9,24,25]. Oxidoreductases are frequently used for the synthesis of carbonyl compounds, hydroxy acids, amino acids and chiral alocohols, which are often difficult to prepare [26]. Of particular interest, nicotinamide-dependent alcohol reductases are employed for the preparation of deuterium or tritium labeled compounds, production of dihydroxyacetone and as tools for enzymatic analysis of serum lipids [27]. Similarly, polysaccharide-modifying enzymes such as the starch modifying enzymes are of considerable interest to industry. Therefore, a significant number of metagenome searches have identified polysaccharide-modifying genes [5•,8,10,16•,28,29]. Further, the isolation of enzymes useful for the production of bulk chemicals [30], proteases [31] and nitrilases [32] has been reported. Metagenome searches have also focused on the isolation of genes involved in vitamin biosynthesis. Interest in 2,5-diketo-D-gluconic acid synthesis is related to a new biotechnological process for the production of vitamin C (ascorbic acid) using glucose as a substrate [25]; and interest in biotin biosynthesis genes is linked to the construction of biotin overproducing bacteria for large-scale fermentation of this vitamin [33]. Finally, the isolation of genes encoding for novel therapeutic molecules is a very valuable area of research [34–38]. The genes of interest within these searches are often type I and type II polyketide synthases (β-ketoacyl synthetases) [12,39], which are key genes involved in the synthesis of polyketide antibiotics by Gram-positive *Streptomyces* and are often part of large biosynthetic gene clusters [40].

Recent discoveries on the physiology of non-cultured microbes and their significance for biogeochemical cycles

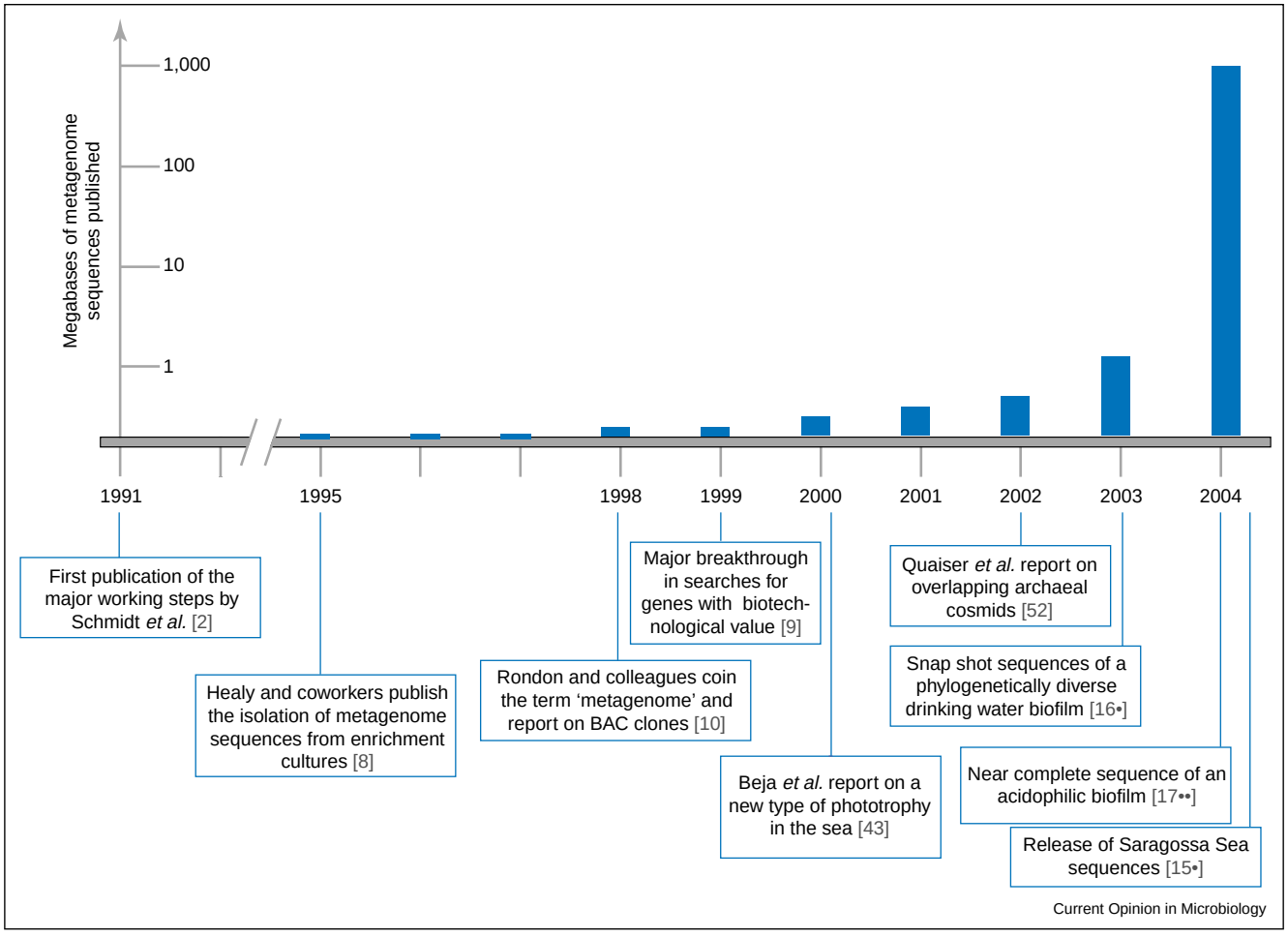
Sequence analysis of large insert libraries with environmental DNA combined with genetic and functional analysis has the potential to provide significant insight into the genomic potential and ecological roles of cultured and uncultured microbes (Figure 1). The importance of this potential for understanding complex environments can be estimated by the following six very recent examples. First, bacteriorhodopsins capable of generating a chemiosmotic membrane potential in response to light have been demonstrated only for halophilic archaea [41]. However, recent analysis of genome fragments recovered directly from marine bacterioplankton suggested the presence of a new bacterial rhodopsin, proteorhodopsin [4,42]. Biochemical and biophysical analyses of this γ-proteobacterial rhodopsin protein expressed heterologously in *E. coli* and analyses of the native protein present in ocean surface waters demonstrated its ability to function as a light-driven proton pump. The discovered widespread distribution of proteorhodopsin genes among divergent marine bacterial taxa [42], the high abundance and spectra adaptation of proteorhodopsin proteins to different habitats combined with the genetic and biophysical data indicate that proteorhodopsin-based bacterial

phototrophy is a globally significant oceanic microbial process [4,11,43–46].

Second, culture-independent partial or nearly complete recovery of microbial genomes from an environmental sample by an extended random shotgun-sequencing approach offers a highly intriguing approach to study natural microbial communities. A recent example gave significant insights into the community structure and the metabolism of a natural acidophilic biofilm growing on the surface of a flowing acid mine drainage [17••]. This was mainly possible through reconstruction of the microbial genomes present in this niche. For this purpose the near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II were reconstructed. A detailed analysis of these genomes allowed pathway reconstruction of carbon fixation and energy generation and provided insights into survival strategies in the extreme acidic environment; for example, genes for biosynthesis of isoprenoid-based lipids and for a variety of proton efflux systems were identified. However, this information has to be confirmed by biochemical and biophysical approaches.

Third, another field for which significant insights in structure, metabolism and gene expression of mixed microbial communities are of high medical interest are biofilms of indwelling medical devices. Recently, Wenderoth and colleagues [47] investigated the community composition of biliary stent biofilms by a cultivation-independent approach, i.e. PCR amplification of 16S rRNA/rDNA, SSCP (single-stranded confirmation polymorphism) fingerprinting of the amplificons and sequencing the major SSCP species. Using this approach, novel uncultured bacteria were identified as the major members of biliary stent biofilms and a sequential colonization process of the stent surface was observed, with *Pseudomonas aeruginosa* being the pioneer colonizer followed by *Klebsiella pneumoniae* and an uncultured microbe [47].

Figure 2



Timescale of metagenomic-derived and published DNA sequences. The timescale ranges from 1991, the initial outline of the major working steps [51], to the first mapping of archaeal comids in 2002 [52] and the snap shot sequence analysis of the Sargasso Sea published earlier this year [14].

Fourth, another example is the analysis of the complex metagenome of model biofilms growing on rubber-coated valves within drinking water networks using the cultivation-independent approach. This analysis revealed significant insights into phylogenetic, catabolic and metabolic abilities of the analyzed microbial community [16•]. Concerning the potential health risk of the studied biofilm, no DNA or protein sequences directly linked to pathogenic traits were identified.

Fifth, the major sequencing approach of the Sargasso Sea has identified a by far greater number of DNA sequences than described in any of the above cited references [15•,48]. This study is most likely to open up the possibility of new investigations of the microbial world and of its evolution. However, it will require new bioinformatics tools and it also highlights how generalist databases such as Genbank or EMBL are not adapted to the overflow of new sequences. In brief, the functions of these many novel sequences and their significance for the ecosystems need to be elucidated over the next decade.

Sixth, most recently mixed microbial communities of interest have been investigated by monitoring gene expression using DNA microarrays [49,50]. This approach however, identifies only those genes transcribed in the analyzed community and is therefore very valuable for the identification of functional genes with relevance to the ecosystem. Though several challenges still remain to be overcome to most effectively apply microarray technology to monitoring gene expression in complex microbial ecosystems (e.g. increasing the sensitivity), the potential and capability of this promising *in situ* technology appears to be highly attractive.

Finally, a completely different but highly intriguing approach was recently reported by Kruger *et al.* [51••]. Cultivation-independent biochemical studies of microbial mats of a northwestern Black Sea shelf, which oxidize methane under anaerobic conditions, resulted in the identification of a prominent nickel-containing protein. Biochemical analyses of this nickel protein isolated from the natural system and its abundance in the methane-oxidizing mat (7% of extracted proteins) indicated that it is likely to catalyze the crucial step in anaerobic methane oxidation (methane activation). This approach is not only remarkable because of the biochemical and ecological findings but also because it first uncovered the function of the novel protein and subsequently applied metagenome technologies to determine the corresponding gene.

Conclusions

It can be expected that the number of novel genes identified through metagenome technologies will exceed the number of genes identified through sequencing individual microbes (Figure 2). However, the challenge now not only lies in accumulating these sequences but moreover in understanding the function of these novel genes and proteins within the microbial niches and their role in the global cycles.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Amann RI, Ludwig W, Schleifer KH: **Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation**. *Microbiol Rev* 1995, **59**:143-169.

2. Schmidt TM, DeLong EF, Pace NR: **Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing**. *J Bacteriol* 1991, **173**:4371-4378.

3. Tsai YL, Olson BH: **Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction**. *Appl Environ Microbiol* 1992, **58**:2292-2295.

4. Beja O, Suzuki MT, Koonin EV, Aravind L, Hadd A, Nguyen LP, Villacorta R, Amjadi M, Garrigues C, Jovanovich SB *et al.*: **Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage**. *Environ Microbiol* 2000, **2**:516-529.

5. • Voget S, Leggewie C, Uesbeck A, Raasch C, Jaeger KE, Streit WR: **Prospecting for novel biocatalysts in a soil metagenome**. *Appl Environ Microbiol* 2003, **69**:6235-6242.

This paper is an example of how successful enrichments and mixed laboratory cultures can be used for the isolation of metagenome-derived sequences. The authors have sequenced and annotated 162 Kb of metagenomic DNA.

6. Knietsch A, Waschkowitz T, Bowien S, Henne A, 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.

7. Entcheva P, Liebl W, Johann A, Hartsch T, Streit WR: **Direct cloning from enrichment cultures, a reliable strategy for isolation of complete operons and genes from microbial consortia**. *Appl Environ Microbiol* 2001, **67**:89-99.

8. Healy FG, Ray RM, Aldrich HC, Wilkie AC, Ingram LO, Shanmugam KT: **Direct isolation of functional genes encoding cellulases from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose**. *Appl Microbiol Biotechnol* 1995, **43**:667-674.

9. Henne A, Daniel R, Schmitz RA, 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.

10. Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C *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.

11. Beja O, Koonin EV, Aravind L, Taylor LT, Seitz H, Stein JL, Bensen DC, Feldman RA, Swanson RV, DeLong EF: **Comparative genomic analysis of archaeal genotypic variants in a single population and in two different oceanic provinces**. *Appl Environ Microbiol* 2002, **68**:335-345.

12. Courtois S, Cappellano CM, Ball M, Francou FX, Normand P, Helynck G, Martinez A, Kolvek SJ, Hopke J, Osburne MS *et al.*: **Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products**. *Appl Environ Microbiol* 2003, **69**:49-55.

13. Henne A, Schmitz RA, Bomeke M, Gottschalk G, 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.

14. Majernik A, Gottschalk G, 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.

15. • Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W *et al.*: **Environmental genome shotgun sequencing of the Sargasso Sea**. *Science* 2004.
This publication is remarkable because of the enormous number of DNA sequences generated and the number of novel genes detected.

16. • Schmeisser C, Stockigt C, Raasch C, Wingender J, Timmis KN, Wenderoth DF, Flemming HC, Liesegang H, Schmitz RA, Jaeger KE *et al.*: **Metagenome survey of biofilms in drinking-water networks**. *Appl Environ Microbiol* 2003, **69**:7298-7309.
This is a landmark publication as it is the first report of partial metagenome sequencing of a phylogenetically highly diverse microbial community. Together with the random snap shot sequences the authors identified more than 100 kb of contiguous environmental DNA sequences.

17. •• Tyson GW, Chapman J, Hugenholtz P, Allen EE, Ram RJ, Richardson PM, Solovyev VV, Rubin EM, Rokhsar DS, Banfield JF: **Community structure and metabolism through reconstruction of microbial genomes from the environment**. *Nature* 2004, **428**:37-43.
This is one of the first publications to present a near-complete set of metagenomic sequences from a mixed but low diversity microbial community.

18. Sebat JL, Colwell FS, Crawford RL: **Metagenomic profiling: microarray analysis of an environmental genomic library**. *Appl Environ Microbiol* 2003, **69**:4927-4934.

19. Marchesi JR, Weightman AJ: **Comparing the dehalogenase gene pool in cultivated alpha-halocarboxylic acid-degrading bacteria with the environmental metagene pool**. *Appl Environ Microbiol* 2003, **69**:4375-4382.

20. Bell PJ, Sunna A, Gibbs MD, Curach NC, Nevalainen H, Bergquist PL: **Prospecting for novel lipase genes using PCR**. *Microbiology* 2002, **148**:2283-2291.

21. Jaeger KE, Dijkstra BW, Reetz MT: **Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases**. *Annu Rev Microbiol* 1999, **53**:315-351.

22. Jaeger KE, Eggert T: **Lipases for biotechnology**. *Curr Opin Biotechnol* 2002, **13**:390-397.

23. Jaeger KE, Reetz MT: **Microbial lipases form versatile tools for biotechnology**. *Trends Biotechnol* 1998, **16**:396-403.

24. Knietsch A, Waschkowitz T, Bowien S, Henne A, Daniel R: **Metagenomes of complex microbial consortia derived from different soils as sources for novel genes conferring formation of carbonyls from short-chain polyols on *Escherichia coli***. *J Mol Microbiol Biotechnol* 2003, **5**:46-56.

25. Eschenfeldt WH, Stols L, Rosenbaum H, Khambatta ZS, Quaite-Randall E, Wu S, Kilgore DC, Trent JD, Donnelly MI: **DNA from uncultured organisms as a source of 2,5-diketo-D-gluconic acid reductases**. *Appl Environ Microbiol* 2001, **67**:4206-4214.

26. Davis BG, Boyer V: **Biocatalysis and enzymes in organic synthesis**. *Nat Prod Rep* 2001, **18**:618-640.

27. Hummel W: **Large-scale applications of NAD(P)-dependent oxidoreductases: recent developments**. *Trends Biotechnol* 1999, **17**:487-492.

28. Richardson TH, Tan X, Frey G, Callen W, Cabell M, Lam D, Macomber J, Short JM, Robertson DE, Miller C: **A novel, high performance enzyme for starch liquefaction. Discovery and optimization of a low pH, thermostable alpha-amylase**. *J Biol Chem* 2002, **277**:26501-26507.

29. Cottrell MT, Moore JA, Kirchman DL: **Chitinases from uncultured marine microorganisms**. *Appl Environ Microbiol* 1999, **65**:2553-2557.

30. Knietsch A, Bowien S, Whited G, Gottschalk G, Daniel R: **Identification and characterization of coenzyme B12-dependent glycerol dehydratase- and diol dehydratase-encoding genes from metagenomic DNA libraries derived from enrichment cultures**. *Appl Environ Microbiol* 2003, **69**:3048-3060.

31. Santosa DA: **Rapid extraction and purification of environmental DNA for molecular cloning applications and molecular diversity studies**. *Mol Biotechnol* 2001, **17**:59-64.

32. DeSantis G, Zhu Z, Greenberg WA, Wong K, Chaplin J, Hanson SR, Farwell B, Nicholson LW, Rand CL, Weiner DP *et al.*: **An enzyme library approach to biocatalysis: development of nitrilases for enantioselective production of carboxylic acid derivatives**. *J Am Chem Soc* 2002, **124**:9024-9025.

33. Streit WR, Entcheva P: **Biotin in microbes, the genes involved in its biosynthesis, its biochemical role and perspectives for biotechnological production**. *Appl Microbiol Biotechnol* 2003, **61**:21-31.

34. Wang GY, Graziani E, Waters B, Pan W, Li X, McDermott J, Meurer G, Saxena G, Andersen RJ, Davies J: **Novel natural products from soil DNA libraries in a streptomycete host**. *Org Lett* 2000, **2**:2401-2404.

35. Brady SF, Chao CJ, Clardy J: **New natural product families from an environmental DNA (eDNA) gene cluster**. *J Am Chem Soc* 2002, **124**:9968-9969.

36. Brady SF, Clardy J: **Synthesis of long-chain fatty acid enol esters isolated from an environmental DNA clone**. *Org Lett* 2003, **5**:121-124.

37. Brady SF, Chao CJ, Handelsman J, Clardy J: **Cloning and heterologous expression of a natural product biosynthetic gene cluster from eDNA**. *Org Lett* 2001, **3**:1981-1984.

38. MacNeil IA, Tiong CL, Minor C, August PR, Grossman TH, Loiacono KA, Lynch BA, Phillips T, Narula S, Sundaramoorthi R *et al.*: **Expression and isolation of antimicrobial small molecules from soil DNA libraries**. *J Mol Microbiol Biotechnol* 2001, **3**:301-308.

39. Piel J: **A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of Paederus beetles**. *Proc Natl Acad Sci USA* 2002, **99**:14002-14007.

40. Challis GL, Hopwood DA: **Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species**. *Proc Natl Acad Sci USA* 2003, **100 (Suppl 2)**:14555-14561.

41. Gartner W, Losi A: **Crossing the borders: archaeal rhodopsins go bacterial**. *Trends Microbiol* 2003, **11**:405-407.

42. de la Torre JR, Christianson LM, Beja O, Suzuki MT, Karl DM, Heidelberg J, DeLong EF: **Proteorhodopsin genes are distributed among divergent marine bacterial taxa**. *Proc Natl Acad Sci USA* 2003, **100**:12830-12835.

43. Beja O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL *et al.*: **Bacterial rhodopsin: evidence for a new type of phototrophy in the sea**. *Science* 2000, **289**:1902-1906.

44. Beja O, Spudich EN, Spudich JL, Leclerc M, DeLong EF: **Proteorhodopsin phototrophy in the ocean**. *Nature* 2001, **411**:786-789.

45. Sabehi G, Massana R, Bielawski JP, Rosenberg M, Delong EF, Beja O: **Novel Proteorhodopsin variants from the Mediterranean and Red Seas**. *Environ Microbiol* 2003, **5**:842-849.

46. Man D, Wang W, Sabehi G, Aravind L, Post AF, Massana R, Spudich EN, Spudich JL, Beja O: **Diversification and spectral tuning in marine proteorhodopsins**. *EMBO J* 2003, **22**:1725-1731.

47. Wenderoth DF, Ferslev B, Macarri G, Molinari G, Lunsdorf H, Timmis KN: **Leitbakteria of microbial biofilm communities causing occlusion of biliary stents**. *Environ Microbiol* 2003, **5**:859-866.

48. Falkowski PG, De Vargas C: **Shotgun sequencing in the sea: a blast from the past?** *Science* 2004.

49. Dennis P, Edwards EA, Liss SN, Fulthorpe R: **Monitoring gene expression in mixed microbial communities by using DNA microarrays**. *Appl Environ Microbiol* 2003, **69**:769-778.

50. Brzostowicz PC, Walters DM, Thomas SM, Nagarajan V, Rouviere PE: **mRNA differential display in a microbial enrichment culture: simultaneous identification of three cyclohexanone monooxygenases from three species**. *Appl Environ Microbiol* 2003, **69**:334-342.

51. •• Kruger M, Meyerdierks A, Glockner FO, Amann R, Widdel F, Kube M, Reinhardt R, Kahnt J, Bocher R, Thauer RK *et al.*: **A conspicuous nickel protein in microbial mats that oxidize methane anaerobically**. *Nature* 2003, **426**:878-881.

This publication is remarkable as it identifies the functional role of a novel protein involved in the global carbon (methane) cycle and it identifies the DNA sequence only subsequently.

52. Quaiser A, Ochsenreiter T, Klenk HP, Kletzin A, Treusch AH, Meurer G, Eck J, Sensen CW, Schleper C: **First insight into the genome of an uncultivated crenarchaeote from soil**. *Environ Microbiol* 2002, **4**:603-611.