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Advances in Recovery of Novel Biocatalysts from Metagenomes

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Key Words
Metagenomics · Biocatalyst · Functional screening · Heterologous host

Abstract
Metagenomics has accelerated the process of discovery of novel biocatalysts by enabling scientists to tap directly into the entire diversity of enzymes held within natural microbial populations. Their characterization has revealed a great deal of valuable information about enzymatic activity in terms of factors which influence their stability and activity under a wide range of conditions. Many of the biocatalysts have particular properties making them suitable for biotechnological applications. A diverse array of strategies has been developed to optimize each step of the process of generating and screening metagenomic libraries for novel biocatalysts. This review covers the diversity of metagenome-derived enzymes characterized to date, and the strategies currently being developed to optimize discovery of novel metagenomic biocatalysts.
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Introduction

Prokaryotes are able to grow in a vast range of environments, from alkaline lakes to hydrothermal vents, indicating that they carry enzymes which are stable and active under these extreme conditions [Grant et al., 2004; Purcarea et al., 2001; Rothschild and Mancinelli, 2001]. These biocatalysts are of interest because they have potential applications in industrial processes, but the majority of microorganisms from such environments, although active, are usually recalcitrant to cultivation [Amann et al., 1995; Ouverney and Fuhrman, 2000]. The fact that traditionally biocatalysts could only be obtained from bacterial isolates was one of the main limitations to the widespread application of biocatalysts in industry [Leresche and Meyer, 2006].

The field of metagenomics developed from advances made in DNA extraction and cloning from environmental samples [Schmidt et al., 1991; Tsai and Olson, 1992]. Around the same time, the application of molecular techniques revealed that prokaryotic diversity had been greatly underestimated and it became apparent that there remained a great deal of biocatalytic potential locked within the unculturable majority of prokaryotes [Amann et al., 1995; Handelsman et al., 1998; Hugenholtz et al., 1998; Streit and Schmitz, 2004]. Current computed estimates of

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Table 1. Amylolytic clones from metagenomic DNA

Library details					Amylolytic clones		Reference
source	vector	clone number	average insert size	total size	total number	level of characterization	
Soil	BAC	3,648	27 kb	100 Mb	8	restriction analysis	Rondon et al., 2000
Various	plasmid	50,000	not specified	not specified	3	protein purification, pH, °C	Richardson et al., 2002
Soil	cosmid	1,532	32.5 kb	49.8 Mb	1	sequencing	Voget et al., 2003
Soil	plasmid	30,000	3.5 kb	105 Mb	1	protein purification, pH, °C	Yun et al., 2004
Cow gut	phage λ vector	14,000 screened	5.5 kb	77 Mb screened	1	activity of crude extract	Ferrer et al., 2005b, 2007

soil diversity are in the range of a million species per 1 g of soil [Curtis and Sloan, 2005; Gans et al., 2005]. Metagenomics now enables access to the biocatalytic potential of unculturable microorganisms [Lorenz and Eck, 2005; Steele and Streit, 2005].

Novel Enzymes from Metagenomes

Metagenomic libraries have been screened for a wide range of biocatalysts [Streit et al., 2004]. Their level of characterization depends strongly on the final application of the biocatalyst. For example, when analyzing metagenomic genes involved in biotin synthesis, the level of enzyme activity is important, and when analyzing metagenomic esterases or cellulases, their stability under a wide range of conditions is as important as their level of activity and substrate range. Many metagenomic biocatalysts, as well as displaying novel substrate/product ranges, are also often highly stable under extreme conditions and therefore have potential applications in industries including the food industry and the production of fine chemicals.

Polysaccharide Degrading/Modifying Enzymes

Agar is used as a solidifying agent in molecular biology research and as an additive in the food industry. Agarases are the enzymes which can liquify agar and they can be divided into α and β agarases, depending on whether they cleave the α-L-(1,3) or the β-D-(1,4) linkages of the polymer. The majority of research into agarases concentrates on those found in cultured bacteria belonging to a limited number of phyla and classes, and mainly isolated from marine environments [Michel et al., 2006]. However, the screening of a soil metagenomic library containing 1,532 clones revealed a total of 4 agarolytic clones containing 12 agarase genes [Voget et al., 2003]. This highlights the potential of using metagenomics to investigate environments for novel biocatalysts which are normally detected in isolates from unrelated environments.

Amylases have many industrial applications, especially in the detergent and food industries, and so far this has been limited to those obtained from cultured microorganisms. As the industrial processes require amylases which are particularly stable over a wide range of pH and temperature conditions, amylases have been predominantly isolated from extremophiles [Pakchung et al., 2006; Rothschild and Mancinelli, 2001]. To date there have been five articles reporting the detection and characterization of novel amylolytic enzymes from metagenomic DNA libraries [Ferrer et al., 2005b; Richardson et al., 2002; Rondon et al., 2000; Voget et al., 2003, Yun et al., 2004] (table 1). Of the 14 amylolytic clones, only 4 have been purified and characterized [Richardson et al., 2002; Yun et al., 2004]. One of the characterized amylases, from a soil metagenome, displayed interesting characteristics in that it is stable and active under alkaline conditions, with a pH optimum at pH 9.0, a characteristic required of amylases in detergents [Rothschild and Mancinelli, 2001; Yun et al., 2004]. The other three amylases were active under acidic pH and high temperature conditions, and these were then subjected to gene reassembly in order to obtain one enzyme displaying optimal properties [Richardson et al., 2002]. Gene reassembly and directed evolution are two methods used to modify natural enzymes at the genetic level in order to optimize them [Chatterjee and Yuan, 2006; Jaeger et al., 2001].

Among many biotechnological applications, cellulases are used in the generation of bioethanol as well as being applied for the polishing and finishing of textiles [Lynd et al., 2002; Ragauskas et al., 2006]. Functional screening of a soil metagenomic library for cellulases revealed a to-

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tal of 8 cellulolytic clones, one of which was purified and characterized [Voget et al., 2006]. Despite the fact that this library had been generated from a soil sample collected from a non-extreme environment, the cellulase displayed a high level of stability over a broad pH range, up to pH 9, it was stable at 40°C for up to 11 h and it was highly halotolerant being active and stable in 3 M NaCl [Voget et al., 2006]. The use of cellulases in detergents requires that they are stable under alkaline conditions [Rothschild and Mancinelli, 2001]. In some cases, thermotolerant cellulases are stabilized at high temperature by high salt concentrations [Bronnenmeier et al., 1995; Liebl et al., 1996]. Metagenomic screening of extreme environments, soda lakes in Africa and Egypt, detected more than a dozen cellulases, some of which displayed habitat-related halotolerant characteristics [Grant et al., 2004; Rees et al., 2003].

One of the earliest articles presenting metagenome-derived biocatalysts reported the detection of cellulases from a thermophilic, anaerobic digester fueled by lignocellulose [Healy et al., 1995]. In this study, 12 cellulolytic clones were detected and sequenced. Four of them were characterized further in terms of their temperature and pH optima and shown to be thermotolerant [Healy et al., 1995].

While most metagenomic surveys for novel cellulases concentrate on extreme environments, there is sufficient evidence that non-extreme, and therefore highly genetically diverse, environments also contain a range of cellulases which are highly stable and suitable for industrial applications [Voget et al., 2006].

Chitinases are responsible for the breakdown and recycling of chitin. With several gigatons of chitin being produced annually, it is the second most abundant polymer in nature after cellulose [Howard et al., 2003]. Chitinases have several biotechnological applications including increasing plant resistance to fungal disease [Carstens et al., 2003]. To date, there has been only one metagenomic study targeting chitinases from a marine environment [Cottrell et al., 1999]. They investigated two different marine environments and used two different methods to screen for chitinases which resulted in the detection of many chitinase-producing clones. While the metagenomic approach provides access to chitinases from cultured and uncultured microorganisms, much can still be learnt from investigating cultured microorganisms for novel chitinases [Hoster et al., 2005].

Xylanases are hydrolytic enzymes which break down the plant cell wall polysaccharide xylan, and they have biotechnological applications in the food and agriculture industries as well as in paper and textiles industries [Collins et al., 2005]. Complete hydrolysis of xylan to xylose also involves the activity of xylosidases. There have been three studies detecting xylanases/xylosidases in the metagenomes of diverse environments: an insect gut, a thermophilic, anaerobic digester and waste lagoon of a dairy farm [Brennan et al., 2004; Healy et al., 1995; Lee et al., 2006a]. Screening of the lagoon resulted in the detection and characterization of one xylanase which displayed habitat-related properties in that it was most active at lower temperatures [Lee et al., 2006a]. The 4 xylosidases detected in the digester were not investigated further, but the cellulases found in the same study were thermophilic [Healy et al., 1995]. The metagenomic survey of the insect gut discovered 4 xylanases which were phylogenetically distant from all other known xylanases, suggesting that they had evolved independently [Brennan et al., 2004]. These xylanases are novel and produce unique hydrolysis products. They also displayed habitat-related properties, being most active at acidic pH and with a temperature optimum at 50°C [Brennan et al., 2004]. The microorganisms present in insect gut are difficult to cultivate and it was only through the application of metagenomics that the biocatalysts involved in hydrolysis processes in the insect gut could be obtained and characterized.

Lipolytic Biocatalysts

The development of metagenomics has greatly accelerated the discovery of novel biocatalysts, many displaying unusual properties. For example, since the first reported screen for detection of lipolytic activity in metagenomic clone libraries in 2000, there have been 13 publications reporting a total of 76 esterase- or lipase-positive clones (table 2). The level of characterization of these novel lipolytic genes ranges from DNA restriction and sequencing analysis to determine clone diversity [Rondon et al., 2000; Voget et al., 2003] to detailed biochemical analysis of the purified enzyme [Elend et al., 2006; Ferrer et al., 2005a; Rhee et al., 2005]. Of the 76 lipolytic-positive clones listed in table 2, only 11 have been overexpressed, purified and subjected to detailed biochemical characterization. It is because of the high number of novel enzymes which can be detected by the screening of a single metagenomic library, and the amount of time and effort required to fully characterize one enzyme which causes this bottleneck.

Esterases and lipases have a growing number of applications in biotechnology, in particular in fine chemicals industries because of their enantioselectivity and stere-

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Table 2. Lipolytic clones from metagenomic DNA

Library details					Lipolytic clones			Reference
source	vector	clone number	average insert size[a] kb	total size	total number	1/Mb DNA screened	1/number of clones	
Soil	plasmid	73,000	6.5	474.5 Mb	1	1/474.5 Mb	1/730,000	Henne et al., 2000
	plasmid	180,000	6.5	1,170 Mb	1	1/1170 Mb	1/180,000	
	plasmid	98,000	6.5	637 Mb	2	1/318.5 Mb	1/49,000	
Soil	BAC	3,648	27	100 Mb	2	1/50 Mb	1/1824	Rondon et al., 2000
Soil	cosmid	1,532	32.5	49.8 Mb	1	1/49.8 Mb	1/1,532	Voget et al., 2003
Kenyan soda lakes	phagemid	60,000	6	360 Mb	2[b]	1/180 Mb	1/30,000	Rees et al., 2003
	phagemid	100,000	6	600 Mb	1	1/600 Mb	1/100,000	
Topsoil	fosmid	33,700	35	1,179.5 Mb	8	1/147 Mb	1/4213	Lee et al., 2004
Pond water	plasmid	30,000 screened	3.8	114 Mb screened	11	1/10.4 Mb	1/2,727	Ranjan et al., 2005
Hot spring	fosmid	2,000	20	40 Mb	4	1/10 Mb	1/200	Rhee et al., 2005
Hot spring	fosmid	10,214	40	408.6 Mb	10	1/40.9 Mb	1/1,021	Kim et al., 2005
Deep-sea hypersaline basin	phagemid	4×10^8 total % screened unspecified	5	2,000 Gb % screened unspecified	5[b]	–	–	Ferrer et al., 2005a
Cow gut	phagemid	14,000 screened	5.5	77 Mb screened	11	1/7 Mb	1/1,273	Ferrer et al., 2005b
Mud flats, beach & forest	fosmid	6,000	35	210 Mb	5	1/42 Mb	1/1,200	Kim et al., 2006
Drinking water	cosmid	1,600	35	56 Mb	6	1/9.3 Mb	1/267	Elend et al., 2006, 2007
Soil	cosmid	2,500	35	87.5 Mb	2[b]	1/87.5 Mb	1/2,500	
Korean tidal flat	fosmid	386,000	35	13.5 Gb	6	1/2.25 Gb	1/64,333	Lee et al., 2006b
Soil, compost	plasmid	32,000[c]	3–8	26 Mb	14	1/12 Mb	1/1,500	Lämmle et al., 2007

– = No information available in the respective article.
[a] When the published insert size was expressed as a range, for example 5–8 kb, the average was taken, in this case 6.5 kb.
[b] With enrichment for esterases.
[c] Only 21,000 clones were tested.

oselective properties [Jaeger and Eggert, 2002]. Among the characterized metagenomic esterases found so far are two of the largest esterases known: a 325-kDa esterase from a deep-sea hypersaline anoxic basin, and a 336-kDa octameric esterase from a drinking water biofilm [Elend et al., 2006; Ferrer et al., 2005a]. The esterases from the deep-sea hypersaline basin display habitat-related properties in that they were most active at alkaline pH and displayed higher activities under high pressure conditions [Ferrer et al., 2005a]. The esterases from soil and a drinking water biofilm displayed unusual properties which could not be related to their environment [Elend et al., 2006]. They were highly stable at alkaline pH and displayed unique substrate spectra with EstA3 being able to hydrolyze substrates such as 7-[3-octylcarboxy-(3-hydroxy-3-methyl-butyloxy)]-coumarin, a normally unreactive secondary ester [Elend et al., 2006]. Within this framework, ibu- and ketoprofen-specific enzymes have recently been reported. One of these enzymes specifically acts on R-ibuprofen esters [Elend et al., 2007] and the second enzyme is highly specify for the S-enatiomer of ketoprofen [Yoon et al., 2007]. Both enzymes have been characterized to a very detailed level.

Despite the fact that many interesting novel lipolytic biocatalysts are discovered using metagenomics, the majority of the biocatalysts remain uncharacterized. This

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bottleneck prevents metagenomic research delivering the full potential of biocatalysts to the biotechnology industries.

Vitamin Biosynthesis

Metagenomics has been applied to the search for novel genes encoding the synthesis of vitamins such as biotin and vitamin C [Entcheva et al., 2001; Eschenfeldt et al., 2001]. Seven cosmids were detected in metagenomic libraries obtained after avidin enrichment of environmental samples [Entcheva et al., 2001]. The highest levels of biotin production in this study were detected in a cosmid obtained from forest soil [Entcheva et al., 2001]. In the search for enzymes involved in vitamin C production from glucose, two novel 2,5-di-keto-D-gluconic acid reductases were PCR amplified from the environment [Eschenfeldt et al., 2001]. These reductases displayed higher activity than other known reductases and they were more thermostable, making them ideal for application in biotechnology.

Nitrilases, Nitrile Hydratases and Amidases

Chemical hydrolysis of nitriles requires the presence of strong acids or bases at high temperatures and normally results in low yields. Therefore, there is much interest in using biocatalysts to carry out this reaction instead [Cantarella et al., 2006].

Nitriles can be hydrolyzed directly to carboxylic acid and ammonium by nitrilases or they are first converted to amides by nitrile hydratases and then the amide is converted to the corresponding carboxylic acid and ammonium by amidases [Liebeton and Eck, 2004].

Nitrile hydratases are used for the production of acrylamide and the vitamin nicotinamide [Brady D. et al., 2004]. In one metagenomic study, involving the screening of 3 Gb DNA, 12 novel nitrile dehydratases were detected [Liebeton and Eck, 2004].

In a general screening of a soil metagenomic library for biocatalysts, one amidase-positive clone was detected [Voget et al., 2003]. Amidases are used in the biosynthesis of β-lactam antibiotics, and in a study targeting amidases of the soil metagenome using enrichment, 7 amidase-positive clones were detected, one of which encoded a novel penicillin acylase [Gabor et al., 2004b, 2005].

Nitrilases are quite rare in bacterial genomes and less than 20 were reported prior to the application of metagenomics for their detection in environmental DNA [DeSantis et al., 2002; Podar et al., 2005; Robertson et al., 2004]. Two studies targeting environmental genomes report the detection of more than 337 novel nitrilases [DeSantis et al., 2002; Robertson et al., 2004]. This has dramatically increased the amount of information about nitrilases, and the newly discovered diversity can be applied for the enantioselective production of hydroxy carboxylic acid derivatives [DeSantis et al., 2002; Podar et al., 2005].

Oxidoreductases/Dehydrogenases

A metagenomic study searching for the diversity of microorganisms in the environment capable of utilizing 4-hydroxybutyrate found 5 clones displaying novel 4-hydroxybutyrate dehydrogenase activity [Henne et al., 1999]. In a recent metagenomic study the genes involved in metabolism of poly-3-hydroxybutyrate, a compound being considered as a substitute for fossil fuel-derived polymers, were screened for in environmental libraries [Wang et al., 2006a]. They found novel short-chain dehydrogenases/reductases which had <35% similarity to known enzymes and thus could not have been detected using hybridization-based techniques such as PCR [Wang et al., 2006a].

Alcohol oxidoreductases capable of oxidizing short-chain polyols are useful biocatalysts in industrial production of chiral hydroxy esters, hydroxy acids, amino acids and alcohols [Knietsch et al., 2003b, c]. In a metagenomic study without enrichment, a total of 24 positive clones were obtained and tested for their substrate specificity [Knietsch et al., 2003c]. To improve the detection frequency, an enrichment was performed using glycerol or 1,2-propanediol, and in this study a further 24 positive clones were detected [Knietsch et al., 2003b]. The difference between the two studies was that without enrichment 300,000 clones per library per substrate were screened whereas with enrichment 100,000 clones per library were screened to obtain the same number of positive clones.

Production of Bulk Chemicals

In industrial processes 1,3-propanediol is used for the production of polyester fibers, polyurethanes and cyclic compounds [Knietsch et al., 2003a]. 1,3-Propanediol can be produced from glucose with the limiting step catalyzed by glycerol dehydratase [Knietsch et al., 2003a]. The aim is to overcome this by utilizing natural biocatalysts with higher activities [Nakamura and Whited, 2003]. A metagenomic survey for glycerol hydratases from the environment resulted in 7 positive clones, one of which displayed a level of catalytic efficiency and stability making it ideal for application in the production of 1,3-propanediol from glucose [Knietsch et al., 2003a].

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Antibiotics and Pharmaceuticals

Metagenomics has greatly enhanced the discovery of novel pharmaceutically important small molecules. There have been two studies reporting the successful screening of soil metagenomic libraries for indirubin, a microbial product used in the treatment of diseases including leukemia [Lim et al., 2005; MacNeil et al., 2001].

A range of genes encoding for novel antibiotics have been detected in metagenomic libraries [Brady and Clardy, 2004, 2005a, b; Brady S.F. et al., 2004; Gillespie et al., 2002]. Palmitoylputrescine was detected in metagenomic DNA from a Bromeliad tank [Brady and Clardy, 2004] and an isocyanide-containing antibiotic was found in a soil metagenomic library [Brady and Clardy, 2005a]. Screening of seven different soil metagenomic libraries revealed 11 clones producing long-chain *N*-acyltyrosine antibiotics and analysis of their synthases indicated that 10 of them were novel [Brady S.F. et al., 2004]. In a related study, other antibacterially active long-chain *N*-acyl amino acids and their synthases were detected in a soil metagenome library [Brady and Clardy, 2005b]. Turbomycin A and B, broad-spectrum antibiotics active against Gram-positive and Gram-negative bacteria, were found in a soil metagenomic library [Gillespie et al., 2002]. In a screening of 1,020 clones, 18 were found to produce terragines [Wang et al., 2000]. Altogether, this indicates that there are many novel antibiotics in the environment and metagenomics makes it possible to detect and characterize them. All the metagenome-derived novel antibiotics detected to date have been found in libraries maintained in *Escherichia coli* hosts except the terragines which were in a streptomycete host [Wang et al., 2000].

As well as finding novel antibiotics in metagenomic studies, a lot of information has been gained about the diversity of natural antibiotic resistance mechanisms [Diaz-Torres et al., 2003; Riesenfeld et al., 2004].

One of the earliest metagenomic studies to target antitumor polyketides and their synthases screened a soil library using PCR and found 12 unique nucleotide sequences [Courtois et al., 2003]. In a second soil metagenomic study, 139 polyketide synthase clones were found in 60,000 screened [Ginolhac et al., 2004]. As a greater diversity of polyketide synthases are detected, the range of new compounds which can be produced increases. Since then, metagenomic polyketide studies have concentrated on detecting a vast number of polyketides from symbionts of beetles and from marine environments [Piel et al., 2004a, b, 2005; Schirmer et al., 2005]. Pederin-type polyketides are only found in symbionts [Piel et al., 2004b]. Metagenomic screening strategies for detection of novel natural products can be enhanced by information gained from analysis of isolates. For example, a recent PCR-based screening of cyanobacterial isolates for enzymes of the phosphopantetheinyl transferase superfamily, which are involved in the synthesis of polyketides, generated 140 sequences from which a functional classification was performed in order to find out more about natural product synthesis by cyanobacteria [Copp and Neilan, 2006]. This sort of information can be incorporated into future metagenomic screens for marine natural products.

Proteases

Proteases are hydrolytic enzymes with important application in industry, in particular in detergents and in the food industry [Gupta et al., 2002]. A screening of a soil library, containing 100 Mb DNA, for proteolytic activity found no positive clones [Rondon et al., 2000]. A second metagenomic study in which 100,000 plasmid clones were screened for proteolytic activity found one positive clone, which was determined to be novel by sequencing analysis [Gupta et al., 2002].

To date (June 2007), there are more than 80 articles reporting on a wide variety of metagenome-derived novel biocatalysts. The earliest articles reported mainly the detection of novel biocatalysts and it is still the case in more recent articles that more biocatalysts are detected than are characterized. The majority of all metegenome studies reports on the isolation of hydrolases EC 3-. Given the growing number of enzymes identified, it is an intriguing challenge to now establish biotechnological processes with these enzymes. However, it might be a very optimistic view to expect that metagenome-derived biocatalysts perform in general better than those already implemented in biotechnology processes. To our knowledge, no metagenome-derived enzymes have yet replaced enzymes from known bacterial isolates in any biotechnological application. It is probably more reasonable to expect that only those metagenome enzymes that carry novel and unusual features will end up in large-scale processes. This means that these enzymes should match at least one of the following two criteria: firstly, these enzymes should allow access to novel substrates and thereby generate products of which the synthesis was not possible with the enzymes already available, and secondly the metagenome-derived biocatalysts should function under conditions for which no classical enzymes have yet been identified. Only if we can identify such enzymes will it be of an advantage to implement them in any biotechnological production processes.

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Strategies for Enhancement of Novel Enzyme Discovery

A range of novel enzyme detection strategies have been developed which target different stages in the process of metagenomic library construction and screening.

Selection of Environment

Metagenomic studies fall into three main categories in terms of their selection of environments. The first group select environments naturally enriched for the target biocatalyst such as the search for xylanases in insect gut [Brennan et al., 2004]. The second group select highly genomically diverse environments such as soil and either directly extract and clone the DNA or subject the community first to enrichment and then proceed with extraction and cloning. The third group target extreme environments and search for biocatalysts which are stable under the conditions experienced by the microorganisms in the environment [Ferrer et al., 2005a].

While extreme environments do yield enzymes that catalyze reactions under extreme conditions, the variation may be quite limited, such as alkalophilic enzymes which function only over a narrow alkaline pH range. Also, many microorganisms growing under acidic or alkaline conditions are able to maintain their intracellular pH around neutral, meaning that only the properties of extracellular enzymes would reflect environmental conditions. Highly genomically diverse environments which are naturally less extreme and more heterogeneous in terms of being subjected to greater fluctuations on temperature, pH or salinity may provide biocatalysts which show a greater level of stability and activity over a wider range of conditions [Elend et al., 2006; Voget et al., 2006].

One of the most significant developments is the application of metagenomics for the detection of pharmaceutically relevant products (antibiotics, anticancer drugs, etc.) from symbiotic marine environments [Piel et al., 2004b, 2005; Wang, 2006b]. Symbiotic bacteria produce many natural products which were previously inaccessible because of the cultivation restraints of these organisms. The application of metagenomics has overcome this obstacle. However, it has been discovered that bacteria can contain many different pathways for the synthesis of a single natural product and the main challenge now is to identify the correct genes among many homologues [Piel et al., 2005].

It must be noted that choice of an environment enriched for activity of the target biocatalyst does not guarantee the high detection frequency of that biocatalyst. One study targeted the metagenome of the bovine rumen, an environment naturally enriched for amylases, and found one amylase-like clone per 14,000 screened, representing 77 Mb DNA [Ferrer et al., 2005b]. This was much lower than the rate of detection of amylases from soil where one positive clone was found per 12.5 Mb DNA screened [Rondon et al., 2000]. This may be related to the cloning vector used in each case: phagemids were applied in the bovine rumen metagenome study and bacterial artificial chromosomes in the soil study. It has been demonstrated that enzyme activity in plaque-based screens is lower than that detected in whole cell-based screens [Cottrell et al., 1999].

Cloning Strategy

The metagenomic DNA is cloned into either large insert or small insert libraries. Large insert libraries can be generated in cosmids [Courtois et al., 2003; Entcheva et al., 2001] or bacterial artificial chromosomes (BAC) [Rondon et al., 2000] or fosmids [Kim et al., 2006; Rhee et al., 2005]. Small insert libraries are maintained in plasmid vectors including pBluescript SK+ [Henne et al., 1999], pUC19 [Ranjan et al., 2005], pZero-2 [Gabor et al., 2004b] and ZAP phagemid vector [Ferrer et al., 2005a]. In the case of pZero-2, a lethal gene is inactivated by the incorporation of environmental DNA which keeps the level of clones containing a self-ligated vector to a minimum [Gabor et al., 2004b]. Small insert libraries contain on average ten times more clones than large-insert libraries covering the same amount of environmental DNA. While the reduced clone number makes large insert libraries easier to screen, the larger insert size and lower copy number makes detection of weakly expressed foreign genes more difficult [Daniel, 2005].

Sequencing Metagenomic DNA

The amount of information available about environmental genomes has been greatly increased by expansive sequencing projects such as that carried out with DNA collected from the Sargasso Sea [Venter et al., 2004]. Sequence-based analysis of genomic data from marine environmental libraries has revealed an abundance of hydrolytic enzymes including 113 cellulases and 20 lipases [Cottrell et al., 2005; Rhee et al., 2005]. The most abundant cellulase-like gene in the Sargasso Sea metagenomic library is *celM*, but biochemical analysis of a CelM protein revealed that it had peptidase activity but no cellulase

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activity [Cottrell et al., 2005]. While sequencing analysis provides a great deal of information, it cannot be used to confirm gene function.

A sequencing-based analysis of a drinking water biofilm resulted in the detection of 21 partial lipase genes [Schmeisser et al., 2003]. A functional-based screening of the same biofilm detected 6 active lipolytic clones [Elend et al., 2006]. The lower number of lipolytic clones detected may be related to limitations in heterologous gene expression.

One of the most common uses of sequence information in metagenomics is to determine whether biocatalysts obtained by functional screening are novel so that biocatalysts that have already been characterized can be excluded from further analysis [Ginolhac et al., 2004].

PCR Detection of Novel Biocatalysts

PCR amplification of novel biocatalyst genes from environmental DNA limits detection to those genes which are genetically similar to already characterized genes. For example, a study of chitinases obtained from a marine environment comparing those from isolated bacteria with those obtained by PCR amplification from the environment revealed that the dominant cellulases detected in both cases were from α-proteobacteria [Cottrell et al., 2000]. As the primers were designed from chitinase sequences from cultured bacteria, this level of similarity between the two studies could be expected. Despite this limitation, this method has been successfully applied for the detection of novel biocatalysts including lipases, chitinases and amylases [Bell et al., 2002; Cottrell et al., 2000; Jiang et al., 2006; Tang et al., 2006]. Combining PCR detection of small conserved regions of biocatalysts with genome walking to obtain upstream and downstream sequences flanking the fragments makes it possible to obtain and characterize the entire gene [Jiang et al., 2006].

Range of Functional Screening Methods

Many of the functional screening methods depend on plating metagenomic clones onto indicator agar and observing activity through color change or the formation of a halo surrounding the colony. Often they are methods derived from classical microbiology where they were previously applied for the analysis of single species rather than for screening of thousands of clones.

In some cases, different laboratories use different screening methods for the detection of the same type of biocatalyst. For example, for the detection of lipolytic clones the range of indicators used include tributyrin, triolein, bactolipid and α-naphtylacetate [Elend et al., 2006; Ferrer et al., 2005a, b; Henne et al., 2000; Rondon et al., 2000].

The functional screening methods all differ in their detection level sensitivity. One metagenomic study targeting chitinases from marine environments compared two functional screening approaches: a plaque assay using an analogue of chitin and a microtiter plate assay with phagemids adsorbed to cells and incubated with the same analogue of chitin [Cottrell et al., 1999]. With the plaque assay, 2 positive clones were detected and with the microtiter plate assay, 432 fluorescing cells were detected. Further analysis of 14 of the wells revealed 13 chitinase-positive clones with a stable phenotype [Cottrell et al., 1999]. The whole cell, microtiter plate assay was much more sensitive than the plaque assay, possibly due to the higher enzyme activity in the unlysed cells.

On the whole, less sensitive functional screening methods detect fewer biocatalysts but they are more active than those detected by more sensitive screens. The optimal screening method required depends whether a wide diversity of functional biocatalysts are required or just the most active.

Laboratory Enrichment Cultures

Searching for novel enzymes in metagenomic libraries often involves screening thousands of clones to find only a few positives. To enhance the likelihood of finding positive clones it is possible to generate a laboratory enrichment culture from the environmental sample material. The aim is to achieve an enrichment of the population carrying the target trait, such as biotin synthesis, esterase or amidase activity, by feeding the appropriate substrates to the sample and incubating them prior to DNA isolation [Elend et al., 2006; Entcheva et al., 2001; Ferrer et al., 2005a; Gabor et al., 2004b]. The enrichment for the target trait is balanced against overall loss of population diversity through cultivation. Both factors lead to an increase in the chance of finding positive clones. One important consideration is that the conditions used for the enrichment, such as temperature and pH range, may be reflected in the temperature and pH optima of the enzymes obtained. When no enrichment step is used then the characteristics of the obtained enzymes may be related to the conditions in their original environment. Enrichment can lead to a 3-fold increase in the detection frequency of positive clones [Knietsch et al., 2003a, b].

In a refinement of the enrichment method which incorporates developments from microbial ecology research, stable isotope labeling and fractionation of the DNA was used to target DNA from the population ac-

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tively metabolizing the labeled substrate and use it to generate the metagenomic library [Friedrich, 2006; Lueders et al., 2004; Schwarz et al., 2006]. In this way, gene detection frequencies were improved 3.8-fold in a metagenomic survey for coenzyme B_{12}-dependent glycerol dehydratases [Schwarz et al., 2006].

Heterologous Gene Expression

Functional screening strategies aim to detect the biocatalytic reaction first and then characterize the gene responsible. In this way, completely novel enzymes can be discovered. This approach requires that the metagenomic DNA be expressed in a heterologous host, usually *E. coli*. As can be expected with an expression-based system, it is limited when factors required for transcription and translation of the metagenomic DNA are not present in the host. A survey of 32 prokaryotic genomes found that only 40% of genes could be expressed using *E. coli* [Gabor et al., 2004a]. Also, even when a gene is expressed, the level of expression can be so low that it cannot be detected using functional screening. Recently, a transposon was developed which enables inducible expression over T7 promoters in both directions [Leggewie et al., 2006]. This transposon could be used to enhance gene expression and increase the likelihood of finding metagenomic enzymes through functional screening.

Improvements in heterologous expression systems for production of complex bacterial products, such as polyketides, help to optimize systems for functional screening in metagenomic research [Wenzel and Müller, 2005].

Selection of Heterologous Host for Functional Screening

It is quite often reported in metagenomic studies that different positive clones are detected when different hosts are used [Li et al., 2005; Wang et al., 2006a]. For example, in a metagenomic screen for D-3-hydroxybutyrate dehydrogenase using both *Sinorhizobium meliloti* and *E. coli bdhA* mutants, a host-specific detection was recorded as only one of 25 clones detected using *S. meliloti* was able to complement *E. coli* and none of the clones found using *E. coli* could be used to complement *S. meliloti* [Wang et al., 2006a].

In a metagenomic study where three different hosts were used to screen a metagenomic library, different positive clones were detected in each of the hosts used: *E. coli*, *Pseudomonas putida* and *Streptomyces lividans* [Martinez et al., 2004]. This clearly indicates that screening of metagenomic libraries can be improved when a range of hosts are used. The heterologous hosts used in metagenomics are *E. coli*, *P. putida*, *S. lividans*, *S. meliloti* and *Rhizobium leguminosarum* [Li et al., 2005; Martinez et al., 2004; Wang et al., 2000; Wexler et al., 2005].

E. coli is used most often as a host for generating metagenomic libraries because it is genetically well defined and easy to transform. With the increase in genome-sequencing projects, many more microorganisms are now genetically defined and could be used as heterologous hosts. Of particular interest are microorganisms with alternative gene expression systems and a wider range of protein secretion mechanisms than *E. coli* as functional screening assays require secretion of the foreign protein.

Substrate-Based Detection Systems

In the SIGEX system the environmental DNA is cloned into a vector containing a *gfp* reporter [Uchiyama et al., 2005]. Clones carrying the catabolic genes are activated in the presence of the target substrate. When the catabolite genes are expressed they in turn activate the *gfp* reporter causing the cell to fluoresce. This, in combination with fluorescence activating cell sorting (FACS), greatly increases the number of recombinant clones that can be screened [Uchiyama et al., 2005]. There are some limitations to this method which include the fact that the catabolite genes have to be cloned in the same orientation as the reporter, transcriptional regulators have to be close to the catabolic genes, there are some catabolic genes which are not induced by the presence of the substrate and finally, the substrate must be transported to the cytoplasm. Despite this, SIGEX is a useful tool and can be developed for detection of genes involved in antibiotic synthesis or catabolism [Yun and Ryu, 2005].

Product-Based Detection Systems

A recent development in detection methods is the ability to detect intracellular biocatalytic activity by linking the product to a reporter gene [Mohn et al., 2006; van Sint Fiet et al., 2006]. One system is designed to detect benzoate and 2-hydroxybenzoate production from their respective aldehydes [van Sint Fiet et al., 2006]. Detection of the products is achieved by combining NahR, a transcriptional activator protein recognizing the products, with reporter and selection systems such as *lacZ* or *tetA*. This system was successfully applied with a detection frequency of 1 in 1 million. The above example can be applied for detection of nitrilases, amidases, aldehyde oxidases and aldehyde benzoates, indicating that detection is not limited to one enzyme but rather to the product

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which may be obtained by different biocatalytic reactions [van Sint Fiet et al., 2006].

Another example of product-related detection of biocatalysis is the development of genetic traps to detect biocatalytic activity [Mohn et al., 2006]. This system was developed for detection of the production of 1,2,4-trichlorobenzene (TCB) from γ-hexachlorocyclohexane (γ-HCH) using a variant of the XylR transcriptional activator which responds to TCB and induces *lacZ* genes cloned downstream of a *Pu* promoter [Mohn et al., 2006]. They demonstrated that it was possible to modify the system using alternative promoters and reporter genes. Modifications to this system, such as application of alternative transcriptional regulators, could expand the range of biocatalysts detected [Konarzycka-Bessler and Jaeger, 2006].

Continuing Search for Novel Biocatalysts

The emphasis in metagenomic research remains more on improving the detection of novel biocatalysts rather than on improving the range of tests used for their biochemical characterization. Metagenomic studies need to broaden and improve the biochemical characterization of novel biocatalysts as well as improve screening and detection strategies. Also, to enable comparison between metagenomic biocatalysts requires the development and application of a range of standard tests, as it has been demonstrated that using different substrates to determine pH and temperature optima can result in a range of catalytic optima being detected [Tang et al., 2006]. Importantly, metagenomic research has to consider the final application of the biocatalysts and the current, specific requirements in biotechnology processes, such as the need for biocatalysts which are active and stable in ionic liquids. Finally, an interesting biocatalytic property can only be detected if the appropriate biochemical tests are developed and applied.

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