The value of microbial diversity

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In the past few years, due to the use of molecular methods, our knowledge of microbial diversity has increased dramatically, not only from a phylogenetic and taxonomic perspective but also from an ecological basis. We now know that microorganisms exist in every conceivable place on Earth, even in extreme environments. Temperature may be the only limitation as to where they can and cannot exist and/or function. As more small subunit rDNA sequence information becomes available there is a real need to start turning the information into knowledge that can be applied to better elucidate and understand structure–function relationships within ecosystems, develop new culturing methods, and discover new products and processes. It has been stated on numerous occasions that the 21st century is the century for biology. Within that context, we must address the real value of microbial diversity.

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Abbreviation
ss small subunit

Introduction

> "By which one sees an unperishable entity in all beings and undivided among the divided then that knowledge is pure. But if one merely sees the diversity of things with their divisions and limitations, without the truth, then that knowledge is merely an ignorance." *The Bhagavad Gita, Chapter XVIII.*

This review addresses the value of microbial diversity with respect to ecophylogeny, function, and benefits and highlights work published between January 1997 and March 1998. Microbial diversity includes the genetic composition of microorganisms, the environment or habitat where they are found, and their ecological or functional role within the ecosystem. Biodiversity is defined "as all hereditarily based variation at all levels of organization, from the genes within a single local population or species, to the species composing all or part of a local community, and finally to the communities themselves that compose the living parts of the multifarious ecosystems of the world" [1].

In 1991, there was a lot of excitement about and questions regarding the agreement made between Merck and Company Incorporated and the Costa Rican National Biodiversity Institute (INBio) for supplying biological samples and information that could contribute to the development of pharmaceuticals derived from Costa Rica's biological resources. Biodiversity prospecting had arrived, but to date, no drugs have been formulated as a result of Merck's biodiversity acquisitions [2]. Since 1992, however, 142 countries have ratified the Convention on Biological Diversity and agreed to promote the conservation, sustainable utilization, and the equitable sharing of benefits from biological diversity at the ecological, organismal, and genetic levels.

The enviromentalists, the public, and policy makers have focused heavily on the need to broaden our understanding of the diverse plant and animal species on this planet. Microbiologists have had to educate these same individuals as to the enormous impact and role microorganisms have in our daily lives, including everything from maintaining the biosphere to improving our life style.

Molecular ecology studies indicate that perhaps 1–5% of microbial species have been isolated. There are several questions we should consider when we discuss the value of microbial diversity. How valid is the assessment of molecular ecology when compared to functional diversity within ecosystems? Are current industrial screens currently well designed to handle the new microbial diversity as defined by unique small subunit rDNA (ssrDNA) sequences? Has sequencing information resulted in knowledge that will increase the amount of microbial products and processes for industries involved in producing commodities such as therapeutics, agrobiologicals, and bioremediation technologies?

Assessing microbial diversity

Prokaryotes are believed to have inhabited Earth for more than 3.5 billion years and yet have remained simple and small throughout their evolutionary history. Their diversity is expressed in terms of physiology and metabolism, while that of the larger eukaryotes is expressed in terms of structure and behavior [3••]. Prokaryotes have optimized their biochemistry for the uptake and utilization of a wide variety of nutrients thereby creating unique pathways and genetic regulation to meet a variety of conditions within nature. Microbial diversity under the best scenarios would examine the number of species within a community and the size of these species populations. Since microbial communities differ in numbers and types, the ratio of individual

community members is subject to both physical and chemical changes in the environment as well as to changes caused by the physiological and metabolic activities of the microorganisms. Microorganisms that are abundant and that can be cultured under certain conditions may develop into dormant and possibly unculturable forms under other environmental conditions. This is why use of PCR, which amplifies small amounts of DNA, such as 16S rDNA, and detects microorganisms occurring in low numbers in the environment is advantageous in assessing microbial diversity.

Microbial ecology studies usually involve the isolation of microorganisms and detection of processes and products within the community by employing biological and chemical assays. Most molecular ecology studies assess the 16S rDNA sequences of prokaryotic microorganisms present in soil, water, and other samples found in nature and/or develop probes to determine if a specific enzyme or product is present in the sample.

Currently there is a big discrepancy between microbial diversity present in a water, soil or other sample collected in nature when assessed by viable plate count technology, direct epifluorescence microscopic counts and ssRNA phylogenetic analysis. Hattori and his associates [4•] address this question and conclude that the discrepancy reflects the unknown physiological status or forms of bacteria residing in the soil. Standard culture techniques using either agar or broth have been limiting for isolating new or unusual strains. Even slight adjustments to media, gas phase or temperature result in only a minimal increase in the number of isolates [4•]. Dunbar *et al.* [5•] demonstrated that any deviation from the original environmental parameters during cultivation can alter the community structure through the imposition of new selective conditions. They compared the diversity of isolates obtained by direct plating on agar to the diversity of those obtained from liquid batch cultures for their ability to degrade 2,4-dichlorophenoxyacetate. The results indicated that a new community structure evolved, which may not have accurately reflected the original microbial community structure with respect to diversity and distribution of catabolic pathways in nature.

It may not be enough to know who is out there. One needs to assess the metabolic plasticity and regulation of the microbial community in order to better understand how the community functions under different conditions. This has been recently examined by Finlay *et al.* [6••], who studied the seasonal effects and changes in pond bacteria and their metabolism. They concluded that microbial activity and diversity are both a part of and inseparable from pond ecosystem function, and that concepts such as redundancy of microbial species and the 'value' of conserving biodiversity at the microbial level have little meaning. Carbon fixation and nutrient cycling appeared to be regulated by 'complex reciprocal' physical, chemical, and microbial interactions. The microbial population or diversity is an integral part of the ecosystem's function rather than a result of reacting to or upon ecosystem function. Little is known about the extent and function of microbial diversity in soils since microbial ecology has been separated from general ecology; funding for any relevant research linking microecology to macroecology has been limited despite the knowledge of fungal and bacterial systems and their importance to plant systems in the rhizophere [7].

Interpretation of microbial diversity

Molecular methods have changed the way we classify and examine microorganisms in their ecosystems and the way we screen for novel products and processes. Colwell [8•] gives a brief summary of the techniques being employed today for studying microbial diversity. There are methods that address taxonomic diversity, such as the amplification of ssrDNA genes from environmental samples, as well as methods used to study community diversity, such as the use of randomly amplified polymorphic DNA (RAPD), which allows for screening and comparison of microorganisms in different sample areas over time rather than identification of individual microorganisms. Terminal restriction fragment length polymorphism (T-RFLP) appears to be a powerful tool for assessing the diversity of complex bacterial communities and for rapidly comparing the community structure and diversity of different ecosystems [9]. Functional genes, such as *recA*, have been shown to be excellent phylogenetic markers. In most cases, there is good agreement between the inferred phylogenetic trees formed by the 16S rRNA and *recA* gene families (SJ Sandler *et al.*, personal communication).

The methods and the pitfalls in assessing microbial diversity using PCR-based rDNA analysis have been described [10••]. It is feasible that there can be a 'distorted view of the real world' in employing PCR amplification since bias is introduced with each physical, chemical and biological step of this technology. For example, the choice of primers and number of cycles of replication can bias the amplification of environmental ssrDNA clones, chimeric molecules can be formed during PCR, or soil components such as humic acid can inhibit the polymerase reaction (see Table 1). Separate phylogenic analysis of short sequence domains has identified chimeras of ssrDNA genes of uncultivated species [10••]. Even with the use of the CHECK_CHIMERA program developed by the Ribosomal Database Project (RDP) and other developed methods, which rely on determining nearest neighbors of different sequence domains for their ability to detect artificially generated ssrDNA chimeras from authentic RDP sequences, chimeras may not be detected because the reliability of all these methods decreases when the parental sequences which contribute to chimera formation are more than 82–84% similar [11].

Table 1

Some pitfalls of PCR amplification in determining microbial diversity present in environmental communities.*

Problems	Factors contributing to problem
Inhibition of PCR amplification	Humic acids and humic substances
Differential PCR amplification	Quantification of microbial communities from analysis of 16S rDNA clone libraries is not possible due to lack of information on genome size and rRNA gene copy number.
Formation of PCR artefacts	Chimeras can be generated between two different DNA molecules with high sequence similarity (homologous genes). Formation of deletion mutants occurs in PCR products due to templates containing stable secondary structures. Formation of point mutants takes place due to intrinsic misincorporation rate during strand synthesis which can lead to base substitutions. There is a minor problem unless mutants are located at sites selected as a probe target or when small differences in sequence are used in strain discrimination.
Contaminating DNA	Target sequence is contaminated, resulting in amplification in negative controls without external DNA being added and co-amplification in experimental reactions.
16S rRNA sequence variations due to rRNA operon heterogeneity	The 16S rRNA genes of some Eubacteria and Archaea reflect the occurrence of interspecific and and intraspecific rRNA operon heterogeneities which interfere with the analysis of 16S rDNA clone libraries or gel electrophoresis patterns derived from environmental samples.

*Adapted from [10••].

In a recent study involving actinomycete species, the level of occurrence of chimeric sequences after 30 cycles of PCR amplification was 32% [12]. Many artificial sequences may have been deposited in the public databases without being noticed because there is no fail-safe way to detect all chimeric sequences. Accumulation of artificial sequences may compromise the quality of the databases and lead to wrong phylogenies for microorganisms and a misinterpretation of the extent of microbial diversity.

On the other hand, many workers skilled in the art of PCR analysis of environmental samples have addressed these problems and provided some solutions [10••]. Other researchers have fine-tuned the algorithms for comparative relational properties of *Methanococcus jannaschii*, an Archaea, to three members of the Eubacteria (*Haemophilus influenzae*, *Mycoplasma genitalium* and *Synechocystis* sp) [13•]. They were able to infer sequence similarity to proteins from other species and predict a function at a general level for a good number of the gene products.

We know from several researchers that microorganisms can exist either in an active form or in a dormant yet persistent form. Hattori *et al.* [4•] concluded that the ratio of viable counts to direct counts reflects the ratio between the numbers of the active (dividing) cells and the 'quiescent cells', and that most bacteria in soil are in the latter form. His phylogenetic analysis of 0.5 kb of 5′-terminal region of 16S rDNA from cultivated isolates showed they were largely diverse. Other studies using 16S rRNA gene sequences indicate that often what may appear to be specific to one habitat can exist in another totally unrelated habitat; for example, the acidobacterium group is found in Sunset Crater Casnino Desert soils, Carolina Bay marine sediments, and fresh water sediments [14,15].

Mycologists estimate that there are 1.5 million species of fungi of which only 72,000 species have been isolated or described [16]. Where are the other 1.428 million unknown fungi? Part of the answer is that there are not many mycologists in the world today and not much work has been done in unique geographical regions or habitats. For example, Hawksworth [16] believes that the largest untapped source of novel fungi species is associated with insects.

The tropics are considered to be richer in microbial species diversity than boreal or temperate environments. When a concentrated effort was directed towards isolating fungi from tropical samples, the numbers increased in direct proportion to the time spent in intensive exploration; 75 leaf dwelling ascomycetes were isolated from one forest in Kenya from which 59 (79%) new species were identified [16]. Despite the increase of microbial species being documented by ssrRNA sequencing, less than 100 species of fungi are sequenced each year even though 1800 new fungi are isolated each year.

Others microbiologists and mycologists feel that there is an equal amount, if not more, microbial diversity in the deserts. Actinomycetes with motile spores appear to be widely distributed in littoral zones and arid environments. Over 500 isolates of a thalloid actinomycete with a motile spore, which is morphologically similar to *Geodermatophilus* but has a unique fatty acid methylester (FAME) signature, were isolated from the Mojave Desert [17]. Sequence analysis of the 16S rDNA of two of these isolates and the inferred phylogenetic tree showed that they form a deep branch within the *Frankia–Geodermatophilus–Sporichthya–Adicothermus* cluster, joining with only one distant relative which was isolated from the Namib Desert in Africa. Phaff reviewed his

lifelong work on the association of yeasts with cactus necroses (H Phaff, abstract S47, Society for Industrial Microbiology Annual Meeting, Reno, Nevada, August 1997) and concluded that the composition of yeast communities found in cactus necroses is affected by the chemical composition of the cactus tissue and the insect vectors that use the rotting material as a habitat for their own reproduction. Most of the yeast species associated with cactus necroses are unique and cactus specific.

Microbial ecophylogeny

Deep in the surface of the earth, new species are being identified that survive for long periods of time without growing or being metabolically active.

Other microorganisms exist in ecosystems based on on rock weathering (JP McKinley, TO Stevens, EL Grossman, Abstract H52C-18, American Geological Union Annual Meeting, San Francisco, California, September 1997). For example, one such ecosystem relies on hydrogen being produced by anaerobic water interacting with basaltic rocks. This reaction was verified under laboratory conditions and helps validate the presence of anaerobic microorganisms in the deep subsurface that exist on hydrogen gas and CO_2 produced from carbonaceous rock.

Geologists are beginning to realize that microorganisms are everywhere and that they may be the major factor in producing oxygen on our planet and in affecting the rock's composition with respect to chemical elements and minerals [18]. There is a growing interest in the interactions of minerals and microorganisms and the data acquired will have an effect on geological research related to biogeochemical dynamics. One such study, by Holman, Perry and myself, has begun to assess the spatial distribution and mapping of microorganisms within fractured basalt and the mineral changes brought about by microbial activity by employing infrared spectroscopy linked to synchrotron beam lines (unpublished data). This study goes beyond the question of who is out there by addressing where are they located inside the rock and whether they are metabolically active or dormant.

The marine deep-sea muds and thermal vents as well as hot springs and geysers located in national parks and volcanoes appear to be favorite sampling sites for many microbiologists today. Their aim is either to sequence 16S rRNA directly from these sites [19•,20••] for phylogenetic analysis and cloning of specific genes or to attempt to isolate new species of extremophilic microorganisms [21]. Pace [19•] discovered, however, that it is not necessary to go to extreme environments to encounter exotic diversity since it is everywhere, even in one's back yard. An example of this is the discovery of mesophilic *Crenarchaeota* in marine and terrestrial environments that were once only thought to be found in high temperature environments [22•,23].

Until two decades ago, diverse life in the deep ocean was not given serious thought. The discovery of deep-sea hydrothermal vents led to the discovery of microbial communities that are very diverse metabolically, physiologically and taxonomically. Within a few hundred square meters, many diverse forms of microbial life exist, such as mesophilic symbionts and nonsymbionts, thermophiles, hyperthermophiles and psychrophiles [24•]. Many of the species isolated have also been found in coastal areas and this may be due to using isolation methods designed for shallow water microorganisms and not employing hydrostatic pressure.

Recent molecular phylogenetic surveys have provided quite a different perspective on Archaean diversity. In marine habitats, on the basis of DNA molecular surveys, Archaea are widely distributed and abundant components of marine plankton. They are also found as mesophiles in fresh water [25] and appear to be related to the marine psychrophilic Archaea. Delong [26] feels that the genomic analysis will 'pave the way' for comparative biochemical and molecular analyses of psychrophilic Archaea and hyperthermophilic Archaea that have not been cultured to date. Other researchers are examining psychrophiles for enzymes that can function at 15°C or lower [20••].

Functional diversity of microorganisms

Some microbiologists believe that it may be more useful to examine functional diversity. A four year study by Dobranic and Zak (personal communication) addressed the issue of linking patterns of taxonomic diversity to ecosystem processes by following the fungal colonization, enzymatic activities, and decomposition of wood at three locations within the northern Chihuahuan Desert. They concluded that similar fungal taxonomic diversity does not result in similar functional diversity and decomposition rates. Shen [27] examined the relationships between agricultural plants and microorganisms such as *Pseudomonas* and *Bacillus* spp. that enable the plant to remain healthy and improve growth yields and researched the mechanisms through which microbially digested organic waste/slurry enhances plant growth and improves soil structure and nutrients.

Another example of functional diversity deals with microbiological processes related to the removal of toxic nitrogen oxides from gaseous pollutants. Growth on nitrogen oxides (NO_x) as the terminal electron acceptor may be a common property of denitrifying bacteria which can utilize NO_x by converting it into N_2 when oxygen becomes limiting [28]. Many denitrifiers produce NO_x reductase and can metabolize NO_x in aerobic and anaerobic conditions. The denitrifiers comprise 130 species within 50 genera and utilize a variety of organic substrates such as sewage sludge, methanol and molasses and inorganic substrates such as thiosulfate, hydrogen and sulfide.

It is now clear that a wide variety of aromatic compounds can be completely degraded by bacteria in the complete absence of oxygen. A major area of interest has been reactions that modify benzene ring substituents, such as dehalogenation and methyl group modification of toluene and cresols. The biotransformations effectively convert toxic compounds to nontoxic derivatives [29•]. Much of the work done to date has been along parallel rather than integrated lines, principally with phototrophic and denitrifying bacteria. What is needed is to coordinate and combine the analytical biochemical and molecular approaches so that the individual threads can be woven together and help us to better understand the metabolic pathways [29•].

Two new groups of iron oxidizing bacteria were isolated a few years ago. One group is photosynthetic bacteria [30] which use solar energy to fix CO_2 to $(CH_2O)_n$ under anaerobic conditions. The electron donor in this reaction is ferrous iron (2+) and the resulting product is ferric iron (3+) in a solid form. The other group is chemolithotrophic bacteria that utilize ferrous iron as the source of energy, while using nitrate (NO_3^-) as the oxidant for anaerobic growth [31]. The bacteria use the energy of the Fe(II)/NO_3-couple to supply energy for carbon fixation and the resulting autotrophic growth. Microbiologists are continually learning that microbial function in ecosystems is as diverse as the microorganisms themselves.

Environmental and economic benefits of microbial diversity

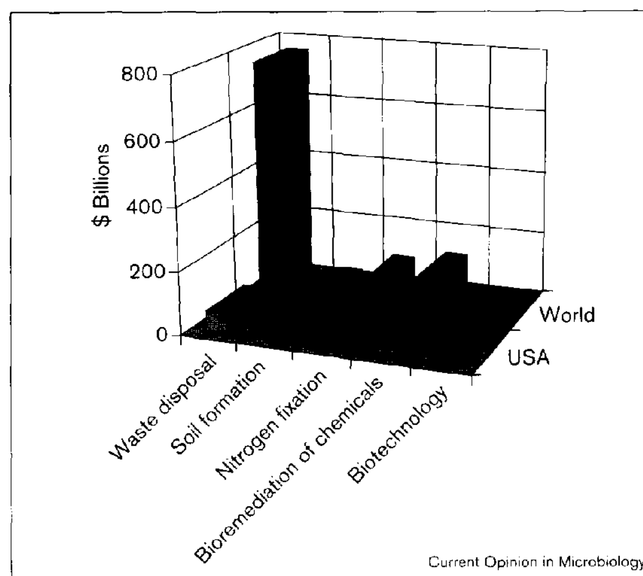
It is difficult to accurately assess all the environmental and economic benefits that are a direct or indirect result of microbial diversity. Only a few are mentioned briefly here.

An economically and environmentally important microbial function is nitrogen fixation. Biological nitrogen-fixation yields in the United States are 14×10^6 tons per year of usable nitrogen with a value of $8 billion and worldwide are 140–170$\times10^6$ tons per year equal to $90 billion [32•].

Bioremediation is increasing in its application as an alternative to pump and treat. It is estimated that nonbiological processes would cost $750 billion over the next 30 years to remediate all of the known hazardous waste sites in the United States [32•]. Using bioremediation the cost would be reduced to $75 billion over the same time period. Worldwide use of bioremediation would cost $14 billion per year compared to use of current technologies at $135 billion per year. Other economically important microbial functions are presented in Figure 1.

Microbial diversity has led to many successes in biotechnology. In the USA, 1993 sales totaled somewhere near $10–$12 billion and by the year 2035 are projected to be near $100 billion [33]. Marine biotechnology has been on a fast track since 1983 when sales were less than $1 million to over $100 million in 1994 [34].

Figure 1



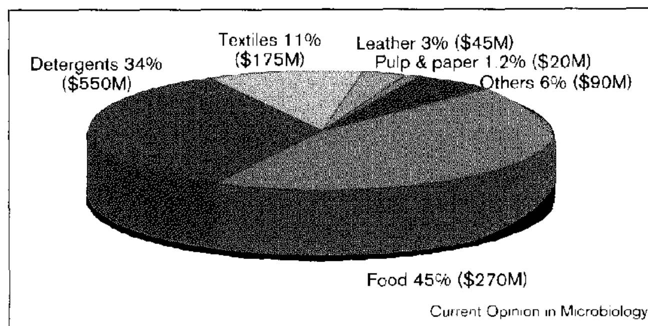
Total estimated economic benefits of microbial diversity. Adapted from [32•] with permission.

Amplification of DNA by PCR using Taq polymerase was one of the more important industrial scale applications of a thermophilic enzyme even though microbiologists began to isolate thermophiles three decades ago. The current market for Taq polymerase is $80 million [35]. A thermophilic cellulase (Cellulase 103, produced by a *Bacillus* sp. that grows at high pH) has been introduced into detergents as an additive to keep cotton clothes looking like new after hundreds of washes in either hot or cold water. The expected market for detergent enzyme additives is around $600 million [36]. Interest in thermophilic proteins came from the hope that they would have applications in biotechnology. The same hope applies to cold-adapted [37] or psychrophilic enzymes. Worldwide sales of industrial enzymes total about $1.6 billion [36]. See Figure 2 for the major customer groups.

Recent interest in microbial surfactants is increasing since they have several advantages over chemical surfactants: lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperatures, pH and salinity, and the ability to be synthesized from renewable feedstocks [38].

Fungi will continue to increase in value with respect to enzymatic properties including the manganese dependent peroxidases [39]. Manganese dependent peroxidases have been reported to depolymerize lignin. There is public concern over negative ecological effects of using chlorine based bleaching methods to 'whiten' paper products. The best biobleaching strains are generally characterized by a predominance of manganese dependent peroxidase activity compared to other liginolytic enzymes. Although

Figure 2



Enzyme market (M, million). 'Others' include diagnostics and therapeutics. 'Food' includes starch processing (11%). Adapted from [36].

the use of white rot fungi in biobleaching has not yet reached industrial scale application, research efforts are being continued because the manganese dependent peroxidases can replace the use of toxic bleaching chemicals.

I have not discussed the dollar value of natural products in this review since that would encompass an entire separate paper. I would, however, like to mention one concern regarding the discovery of new products and processes with respect to where we sample for microbial diversity and that is sample collection fees. Attorneys and political lobbyists are building a case in the United States to have biotechnology companies pay to collect samples from federal lands, such as Yellowstone National Park, where Thomas Brock first isolated *Thermus aquaticus* (from which Taq polymerase was first isolated and commercially produced). Other than Taxol, which is the potent anticancer compound found in the bark of the Pacific yew, a tree found primarily on federal lands, no other commercial product has been documented and acknowledged by biotechnology companies as having been developed after isolation of microbial samples from federal lands [40•]. Hopefully, the question of sampling fees, payments and royalties will not inhibit our quest for new products and processes arising from microbes existing in extreme or unusual habitats.

Conclusions

Several microbial genomes have now been sequenced or are in the process of being sequenced [41]. We still, however, need to examine factors that may cause chimeric sequences and biased amplification, improve experimental conditions to minimize PCR problems, and to develop more sensitive computer programs for the identification of chimeric molecules. With all the sequencing information being accumulated, functional genomics now has the task of turning this information into knowledge so that microbiologists can assess the diversity not only at the molecular level but also at the functional level. With the development of computational biology tools to assess the phylogenetic affiliations and metabolic functions on the basis of comparison of related gene sequences, we should be able to improve our existing isolation methods and begin to develop novel isolation methods which closely reflect the niche from which we are sampling or employ techniques such as 'lazer tweezers' [20••]. We need to apply more geochemistry and earth science to our isolation methods and to lean away from the more traditional methods of isolation with solid and liquid media.

What we don't know about microbial diversity has initiated a renaissance in microbial biological research that will elucidate the structure and function of microorganisms in every conceivable niche on this planet. It appears that perhaps temperature may be the only limit to microbial diversity; for example, new species have been discovered in the deep subsurface and mid-ocean ridges that can extract energy from ancient organic matter or hydrogen and carbon dioxide formed from geochemical reactions within the rock [21].

For microbial ecologists there are many research projects to focus on, especially in the ocean depths, such as the mechanism of symbiont transmission, hyperthermophile distribution and dispersion, genetic exchanges at high temperatures, and the upper temperature limit for life [24•].

The biotechnology and industrial microbiology potential for new products and processes from examining microbial diversity at both the molecular and organismal functional level will increase as more knowledge is deciphered from sequences to assess targets and function, develop new assays, and link with engineers to design new robotic high-throughput screening technology.

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