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HUMAN AND MICROBIAL WORLD

1.1. PROLOGUE

The microbial world is vast, diverse, and dynamic. The Earth hosts over 10^{30} microorganisms, representing the largest component of the planet's biomass. Microbes include bacteria, archaea, mollicutes, fungi, microalgae, viruses, and protozoa, and many more organisms with a wide range of morphologies and lifestyles. All other life-forms depend on microbial metabolic activity. Microorganisms have colonized virtually every environment on earth ranging from deep sea thermal vents, polar sea ice, desert rocks, guts of termites, roots of plants, to the human body. Much as we might like to ignore them, microbes are present everywhere in our bodies, living in our mouth, skin, lungs, and gut. Indeed, the human body has 10 times as many microbial cells as human cells. They are a vital part of our health, breaking down otherwise indigestible foods, making essential vitamins, and even shaping our immune system. While microbes are often feared for the diseases they may cause, other microorganisms mediate the essential biogeochemical cycles of key elements that make our planet habitable. Ancient lineages of microorganisms may hold the key to understanding the earliest history of life on earth.

1.2. INNOVATIONS IN MICROBIOLOGY FOR HUMAN WELFARE

The human society is overburdened with infectious diseases. Despite worldwide efforts toward prevention and cure of these deadly infections, they remain major causes of

Microbes: Concepts and Applications, First Edition. Prakash S. Bisen, Mousumi Debnath, Godavarthi B. K. S. Prasad

human morbidity and mortality. Microbes play a role in diseases such as ulcers, heart disease, and obesity. Over the past century, microbiologists have searched for more rapid and efficient means of microbial identification. The identification and differentiation of microorganisms has principally relied on microbial morphology and growth variables. Advances in molecular biology over the past few years have opened new avenues for microbial identification, characterization, and molecular approaches for studying various aspects of infectious diseases. Perhaps, the most important development has been the concerted efforts to determine the genome sequence of important human pathogens. The genome sequence of the pathogen provides us with the complete list of genes, and, through functional genomics, a potential list of novel drug targets and vaccine candidates can be identified (Hasnain, 2001). The genetic variation inherent in the human population can modulate success of any vaccine or chemotherapeutic agent. This is why, the sequencing of the human genome has attracted not only the interest of all those working on human genetic disorders but also the interest of scientists working in the field of infectious diseases.

1.2.1. Impact of Microbes on the Human Genome Project

Technology and resources generated by the Human Genome Project and other genomic research are already having a major impact on research across life sciences. The elucidation of the human genome sequence will have a tremendous impact on our understanding of the prevention and cure of infectious diseases. The human genome sequence will further advance our understanding of microbial pathogens and commensals and vice versa (Relman and Falkow, 2001). This will be possible through efforts in areas such as structural genomics, pharmacogenomics, comparative genomics, proteomics, and, most importantly, functional genomics. Functional genomics includes not only understanding the function of genes and other parts of the genome but also the organization and control of genetic pathway(s). There is an urgent need to apply high throughput methodologies such as microarrays, proteomics (the complete protein profile of a cell as a function of time and space), and study of single nucleotide polymorphisms, transgenes and gene knockouts. Microarrays have a tremendous potential in

1. Determining new gene loci in diseases
2. Understanding global cellular response to a particular mode of therapy
3. Elucidating changes in global gene expression profiles during disease conditions; and so on.

Increasingly detailed genome maps have aided researchers seeking genes associated with dozens of genetic conditions, including myotonic dystrophy, fragile X syndrome, neurofibromatosis types 1 and 2, inherited colon cancer, Alzheimer's disease, and familial breast cancer.

The Human Microbiome Project (HMP) has published an analysis of 178 genomes from microbes that live in or on the human body. The core human microbiome is the set of genes present in a given habitat in all or the vast majority of humans (Fig. 1.1). The variable human microbiome is the set of genes present in a given habitat in a smaller subset of humans. This variation could result from a combination of factors such as host genotype, host physiological status (including the properties of the innate and

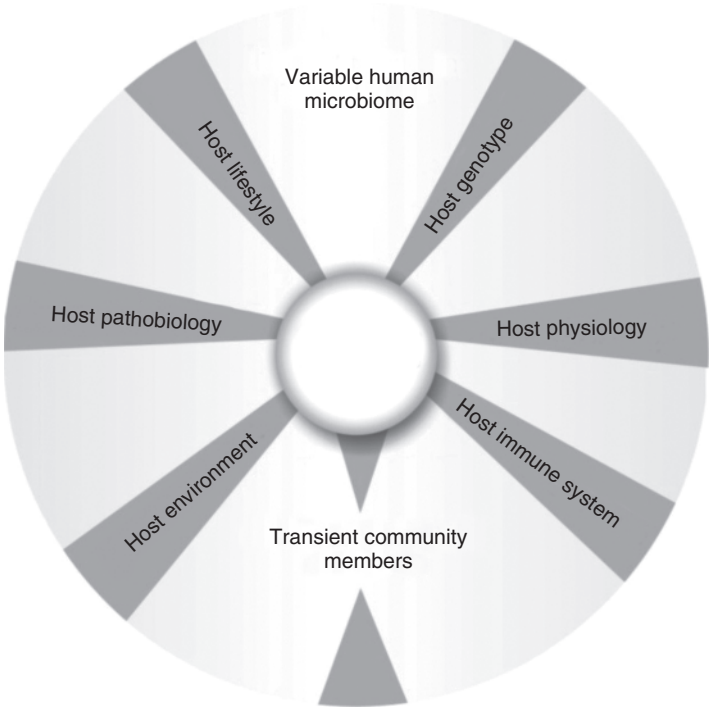

Figure 1.1. The concept of human microbiome.

adaptive immune systems), host pathobiology (disease status), host lifestyle (including diet), host environment (at home and/or work), and the presence of transient populations of microorganisms that cannot persistently colonize a habitat. The gradation in color of the core indicates the possibility that, during human microevolution, new genes might be included in the core microbiome, whereas other genes might be excluded (Turnbaugh et al., 2007). The researchers discovered novel genes and proteins that serve functions in human health and disease, adding a new level of understanding to what is known about the complexity and diversity of these organisms (http://www.nih.gov). Currently, only some of the bacteria, fungi, and viruses can grow in a laboratory setting. However, new genomic techniques can identify minute amounts of microbial DNA in an individual and determine its identity by comparing the genetic signature with known sequences in the project's database. Launched in 2008 as part of the NIH Common Fund's Roadmap for Medical Research, the HMP is a $157 million, five-year effort that will implement a series of increasingly complicated studies that reveal the interactive role of the microbiome in human health (http://www.eurekalert.org). The generated data will then be used to characterize the microbial communities found in samples taken from healthy human volunteers and, later, those with specific illnesses.

Studies were also conducted to evaluate the microbial diversity present in the HMP reference collection. For example, they found 29,693 previously undiscovered, unique proteins in the reference collection; more proteins than there are estimated genes in the human genome. The results were compared to the same number of previously sequenced

microbial genomes randomly selected from public databases and reported 14,064 novel proteins.

These data suggest that the HMP reference collection has nearly twice the amount of microbial diversity than is represented by microbial genomes already in public databases (http://www.ncbi.nlm.nih.gov/genomeprj). One of the primary goals of the HMP reference collection is to expand researchers' ability to interpret data from metagenomic studies. Metagenomics is the study of a collection of genetic material (genomes) from a mixed community of organisms. Comparing metagenomic sequence data with genomes in the reference collection can help determine the novel or already existing sequences (Hsiao and Fraser-Liggett, 2009). A total of 16.8 million microbial sequences found in public databases have been compared to the genome sequences in the HMP reference collection and it was found that 62 genomes in the reference collection showed similarity with 11.3 million microbial sequences in public databases and 6.9 million of these (41%) correspond with genome sequences in the reference collection (http://www.ncbi.nlm.nih.gov/genomeprj).

On the horizon is a new era of molecular medicine characterized less by treating symptoms and more by looking at the most fundamental causes of diseases. Rapid and more specific diagnostic tests will make possible earlier treatment of countless maladies. Medical researchers will also be able to devise novel therapeutic regimens on the basis of new classes of drugs, immunotherapy techniques, avoidance of environmental conditions that may trigger disease, and possible augmentation or even replacement of defective genes through gene therapy.

Despite our reliance on the inhabitants of the microbial world, we know little of their number or their nature. Less than 0.01% of the estimated all microbes have been cultivated and characterized. Microbial genome sequencing will help to lay the foundation for knowledge that will ultimately benefit human health and the environment. The economy will benefit from further industrial applications of microbial capabilities.

Information gleaned from the characterization of complete microbial genomes will lead to insights into the development of such new energy-related biotechnologies as photosynthetic systems and microbial systems that function in extreme environments and organisms that can metabolize readily available renewable resources and waste material with equal facility. Expected benefits also include development of diverse new products, processes, and test methods that will open the door to a cleaner environment. Biomanufacturing will use nontoxic chemicals and enzymes to reduce the cost and improve the efficiency of industrial processes. Microbial enzymes have been used to bleach paper pulp, stone wash denim, remove lipstick from glassware, break down starch in brewing, and coagulate milk protein for cheese production. In the health arena, microbial sequences may help researchers to find new human genes and shed light on the disease-producing properties of pathogens.

Microbial genomics will also help pharmaceutical researchers to gain a better understanding of how pathogenic microbes cause disease. Sequencing these microbes will help to reveal vulnerabilities and identify new drug targets. Gaining a deeper understanding of the microbial world will also provide insights into the strategies and limits of life on this planet, and the human genome sequence will further strengthen our understanding of microbial pathogens and commensals, and vice versa.

Data generated in HMP have helped scientists to identify the minimum number of genes necessary for life and confirm the existence of a third major kingdom of life. Additionally, the new genetic techniques now allow us to establish, more precisely,

the diversity of microorganisms and identify those critical to maintaining or restoring the function and integrity of large and small ecosystems; this knowledge can also be useful in monitoring and predicting environmental changes. Finally, studies on microbial communities provide models for understanding biological interactions and evolutionary history.

1.2.2. Microbial Biosensors

Biosensors are defined as analytical devices combining biospecific recognition systems with physical or electrochemical signaling. They have been used for many years to provide process control data in the pharmaceutical, fermentation, and food-processing industries. The generic system comprises three components: the biospecific interaction, the signal emitted when the target is bound, and the platform that transduces the binding reaction into a machine-readable output signal. Significant progress has been made in developing platforms that exploit recent technological advances in microfabrication, optoelectronics, and electromechanical nanotechnology. Dramatic improvements in device designs facilitated by new tools and instrumentation (Fig. 1.2) have increased biosensor sensitivities by several magnitudes (Tepper and Shlomi, 2010). The designed biosensors facilitate high throughput detection and quantification of chemicals of interest, enabling combinatorial metabolic engineering experiments aiming to overproduce

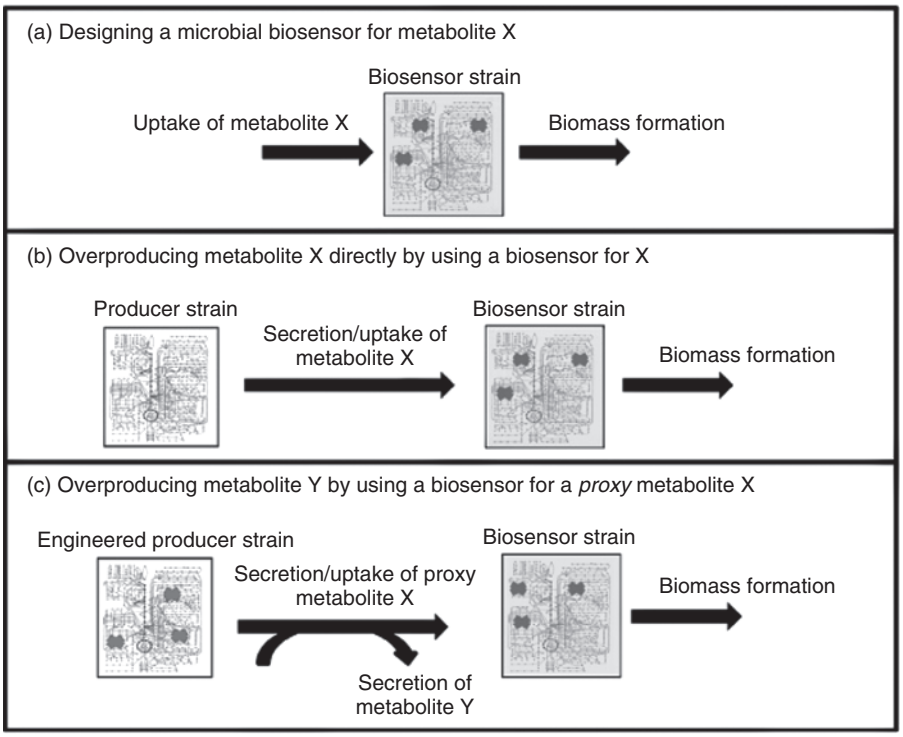

Figure 1.2. Concept of microbial biosensor (Tepper and Shlomi, 2010). (*See insert for color representation of the figure*).

them (http://www.cs.technion.ac.il). New platforms can be arrayed in panels to reduce costs and simplified methods can be employed to detect and validate against numerous hazards.

Majority of the described piezoelectric (PZ) devices are based on immunosensors. Targeting intact bacteria as antibody is relatively simple to immobilize, and entrapped bacterial cells can accumulate a significantly large, detectable mass. Many PZ devices are used in clinical and food pathogen identifications, for example, quartz crystal microbalance (QCM) devices coated with antibody, protein A, or other specific receptor molecules have been applied for a wide range of antibodies, pathogenic organisms (including *Vibrio, Salmonella, Campylobacter, Escherichia coli, Shigella, Yersinia*, viruses, and protozoa) and PCR amplicons (Hall, 2002). The simplicity, flexibility, and utility of the PZ systems in a range of foodborne and clinical applications make it well accepted for QCM format, although the sensitivity of these sensors is suitable only for very dense bacterial cultures. The QCM device coated with specific antibody and integrated into the culture enrichment tube resolves several problems in food safety testing, albeit at the expense of rapidity and cost. There are numerous biological components that can be coupled to mass aggregation and deposition chemistries, largely because the signal amplification required in most ELISA, western hybridization, and dot blot detection systems entails accumulation of precipitated mass in the process of generating a detectable signal. Among the ELISA systems that have been modified for mass sensors is Ag/Abs binding and DNA–DNA hybridization, and indirect, amplified systems such as double anti-angiogenic protein (DAAP) and avidin/streptavidin enzyme-conjugated secondary antibodies.

Microbial biosensors for environmental applications range in their development stages from proof of concept to full commercial availability, and the target detection specificity may fall in one of the following two groups (Bogue, 2003; Rodriguez-Mozaz et al., 2004):

- Biosensors that measure general biological effects/parameters.
- Biosensors for specific detection of target compounds.

The first group of biosensors is aimed to measure integral toxicity, genotoxicity, estrogenicity, or other general parameters of the sample, which affect living organisms. They essentially include whole microorganisms as biorecognition elements. The most-often reported cell-based biosensors include genetically modified bacteria with artificially constructed fusions of particular regulatory system (native promoter) with reporter genes. The presence of effectors (nonspecific stressor such as DNA damaging agents, heat shock, oxidative stress, toxic metals, and organic environmental pollutants) results in transcription and translation of fused target genes, generating recombinant proteins that produce some measurable response. Frequently used reporter genes are *lux* (coding for luciferase) and *gfp* (coding for green fluorescence protein), expression of which correlates with luminescence- or fluorescence-based light emission (Kohlmeier et al., 2006). Colorimetric determination of target gene expression is possible by fusing it to reporter genes coding for β-galalactosidase (*lacZ*) or alkaline phosphatase (*phoA*).

E. coli biosensor capable of detecting both genotoxic and oxidative damage has been developed. This was achieved by introducing two plasmids: fusion of *katG* (gene encoding for an important antioxidative enzyme) promoter to the *lux* reporter genes and *recA* (gene encoding a crucial enzyme for DNA repair) promoter with the *gfp* reporter gene (Mitchell and Gu, 2004). Besides genetically modified microorganisms (also named

bioreporters), some other types of cellular biosensors have also been constructed. An example is the algal biosensor, which functions based on amperometric monitoring of photosynthetic O_2 evolution—the process affected by toxic compounds—was developed by coupling Clark electrode to the cyanobacterium *Spirulina subsalsa* (Campanella et al., 2000). Biosensors for specific determination of chemical compounds frequently contain molecules such as enzymes, receptors, and metal-binding proteins as recognition elements. A number of enzymes have been shown to be inhibited by toxic metals, pesticides, and some other important contaminants such as endocrine disrupting compounds. Limitations for the potential applications of many enzyme biosensors include limited sensitivity and selectivity, as well as interference by environmental matrices Marinšek Logar and Vodovnik, 2007. One recently introduced strategy to overcome the first two of these limitations uses inhibition ratio of two enzymes for the detection of specific compounds. Acetylcholinesterase and urease, coentrapped in the sol–gel matrix with the sensing probe (FITC-dextran), have successfully been used for the detection of Cu, Cd, and Hg (Tsai and Doong, 2005). Besides molecular biosensors, bioreporter cells may also be used for the detection of specific target compounds. A biosensor for nitrate monitoring has been constructed by transformation of plasmid containing nitrate reductase operon fused to *gfp* reporter gene to *E. coli* cells (Taylor et al., 2004).

1.2.3. Molecular Diagnostics

Traditionally, the clinical medical microbiology laboratory has functioned to identify the etiologic agents of infectious diseases through the direct examination and culture of clinical specimens. Direct examination is limited by the number of organisms present and by the ability of the laboratories to successfully recognize the pathogen. Similarly, the culture of the etiologic agent depends on the ability of the microbe to propagate on artificial media and the choice of appropriate media for the culture. When a sample of limited volume is submitted, it is often not possible to culture for all pathogens. In such instances, close clinical correlation is essential for the judicious use of the specimen available. Commercial kits for the molecular detection and identification of infectious pathogens have provided a degree of standardization and ease of use that has facilitated the introduction of molecular diagnostics into the clinical microbiology laboratory. The use of nucleic acid probes for identifying cultured organisms and for direct detection of organisms in clinical material was the first exposure that most laboratories had to explore commercially available molecular tests. Although these probe tests are still widely used, amplification-based methods are increasingly employed for diagnosis, identification, quantization of pathogens, and characterization of antimicrobial-drug-resistant genes.

The tools of molecular biology have proven readily adaptable for use in the clinical diagnostic laboratory and promise to be extremely useful in diagnosis, therapy, and epidemiologic investigations and infection control (Cormican and Pfaller, 1996; Pfaller, 2000, 2001). Although technical issues such as ease of performance, reproducibility, sensitivity, and specificity of molecular tests are important, cost and potential contribution to patient care are also of concern (Kant, 1995). Molecular methods may be an improvement over conventional microbiologic testing in many ways. Currently, their most practical and useful application is in detecting and identifying infectious agents for which routine growth-based culture and microscopy methods may not be adequate

(Fredricks and Relman, 1996; Fredricks and Relman, 1999; Tang and Persing, 1999; Woods, 2001).

Nucleic-acid-based tests used in diagnosing infectious diseases use standard methods for isolating nucleic acids from organisms and clinical material, and restriction endonuclease enzymes, gel electrophoresis, and nucleic acid hybridization techniques to analyze DNA or RNA (Tang and Persing, 1999). Because the target DNA or RNA may be present in very small amounts in clinical specimens, various signal amplification and target amplification techniques have been used to detect infectious agents in clinical diagnostic laboratories (Fredricks and Relman, 1999; Tang and Persing, 1999). Nucleic acid sequence analysis coupled with target amplification is clinically useful to detect and identify previously uncultivable organisms and characterize antimicrobial resistant gene mutations, thus aiding both diagnosis and treatment of infectious diseases (Fredricks and Relman, 1999). Automation and high-density oligonucleotide probe arrays (DNA chips) also hold great promise for characterizing microbial pathogens (Tang and Persing, 1999).

Although most clinicians and microbiologists enthusiastically welcome the new molecular tests for diagnosing infectious diseases, the high cost of these tests is of concern. Molecular methods will be increasingly used for pathogen identification, microbial quantification, and resistance testing.

The use of these detection methods in microbiology laboratories has resolved many problems and has initiated a revolution in the diagnosis and monitoring of infectious diseases. Some microorganisms are uncultivable at present, extremely fastidious, or hazardous to laboratory personnel. In these instances, the diagnosis often depends on the serologic detection of a humoral response or culture in an expensive biosafety level II–IV facility. In community medical microbiology laboratories, these facilities may not be available, or it may not be economically feasible to maintain the special media required for the culture of all of the rarely encountered pathogens. Thus, cultures are often sent to referral laboratories. During transit, fragile microbes may lose viability or become overgrown by contaminating organisms or competing normal flora.

Although direct detection of organisms in clinical specimens by nucleic acid probes is rapid and simple, it suffers from lack of sensitivity. Most direct probe detection assays require at least 10^4 copies of nucleic acid per microliter for reliable detection, a requirement rarely met in clinical samples without some form of amplification. Amplification of the detection signal after probe hybridization improves sensitivity to as low as 500 gene copies per microliter and provides quantitative capabilities. This approach has been extensively used for quantitative assays of viral load HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV) but does not match the analytical sensitivity of target-amplification-based methods such as polymerase chain reaction (PCR) for detecting organisms.

Probe hybridization is useful in identifying organisms that grow slow after isolation from culture using either liquid or solid media. Identification of mycobacteria and other slow-growing organisms such as the dimorphic fungi (*Histoplasma capsulatum, Coccidioides immitis*, and *Blastomyces dermatitidis*), has certainly been facilitated by commercially available probes. All commercial probes for the identification of organisms are produced by Gen-Probe, which uses acridinium-ester-labeled probes directed at species-specific rRNA sequences. Gen-Probe products are available for the culture identification of *Mycobacterium tuberculosis, Mycobacterium avium-intracellulare* complex, *Mycobacterium gordonae, Mycobacterium kansasii, Cryptococcus neoformans*, the

dimorphic fungi (listed above), *Neisseria gonorrhoeae, Staphylococcus aureus, Streptococcus pneumoniae, E. coli, Haemophilus influenzae, Enterococcus* spp., *Streptococcus agalactiae*, and *Listeria monocytogenes*. The sensitivity and specificity of these probes are excellent, and they provide species identification within one working day. Because most of the bacteria listed and *C. neoformans* can be easily and efficiently identified by conventional methods within 1–2 days, many of these probes are not widely used. The mycobacterial probes, on the other hand, are accepted as mainstays for the identification of *M. tuberculosis* and related species.

Nucleic acid techniques such as plasmid profiling, various methods for generating restriction fragment length polymorphisms, and the PCR are making increasing inroads into clinical laboratories. PCR-based systems to detect the etiologic agents of diseases directly from clinical samples, without the need for culture, have been useful in rapid detection of uncultivable or fastidious microorganisms. Additionally, sequence analysis of amplified microbial DNA allows for the identification and better characterization of the pathogen. Subspecies variation, identified by various techniques, has been shown to be important in the prognosis of certain diseases. New advances in real-time PCR promise results that will come fast enough to revolutionize the practice of medicine.

Commercial-amplification-based molecular diagnostic systems for infectious diseases have focused largely on systems for detecting *N. gonorrhoeae, Chlamydia trachomatis, M. tuberculosis*, and specific viral infections [HBV, HCV, HIV, CMV (cytomegalovirus), and enterovirus]. Given the adaptability of PCR, numerous additional infectious pathogens have been detected by investigator-developed PCR assays. This novel, fully integrated device, coupled with appropriate databases, will insure better management of patients, should reduce health costs, and could have an impact on the spread of antibiotic resistance (Boissinot and Bergeron, 2002). Another exciting technology that has demonstrated clinical diagnostic utility is DNA microarray science. DNA microarray enables simultaneous analyses of global patterns of gene expression in microorganisms or host cells. In addition, genotyping and sequencing by microarray-based hybridization have been successfully used for organism identification and molecular resistance testing.

Microbial phenotypic characteristics, such as protein, bacteriophage, and chromatographic profiles, as well as biotyping and susceptibility testing, are used in most routine laboratories for identification and differentiation. Other important advances include the determination of viral load and the direct detection of genes or gene mutations responsible for drug resistance. Increased use of automation and user-friendly software makes these technologies more widely available. In all, the detection of infectious agents at the nucleic acid level represents a true synthesis of clinical chemistry and clinical microbiology techniques.

Ou et al. (2007) have beautifully illustrated an example of how to use whole genome sequence analyses of *Salmonella enterica* paratyphi A and existing comparative genomic hybridization data to design a highly discriminatory multiplex PCR assay that can be developed in any molecular diagnostic laboratory. In a time of overwhelmingly rapid expansion of genomic information, various navigation tools and a recipe for mining the genomic databases to design species, serovar, or pathotype specific PCR assays for accurate identification have been developed (Wenyong et al., 2007).

Molecular methods can rapidly detect antimicrobial drug resistance in clinical settings and have substantially contributed to our understanding of the spread and genetics of

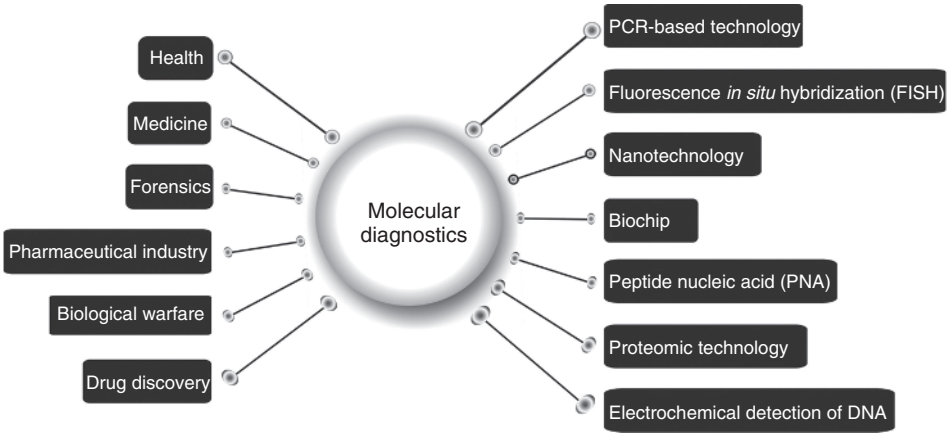

Figure 1.3. Applications and areas of interest related to molecular diagnostics.

resistance. Conventional broth and agar-based antimicrobial susceptibility testing methods provide a phenotypic profile of the response of a given microbe to an array of agents. Conventional methods are slow and fraught with problems, although useful for selecting potential therapeutic agents. The rapid evolution in microbial genomics will transform the process of accurate identification of novel, difficult to culture, or phenotypically indistinguishable pathogens and research and development in diagnostics, or "diagnomics."

Molecular typing methods have allowed investigators to study the relationship between colonizing and infecting isolates in individual patients. Most available DNA-based typing methods may be used in studying infections when applied in the context of a careful epidemiologic investigation. Molecular testing for infectious diseases includes testing for the host's predisposition to diseases, screening for infected or colonized persons, diagnosis of clinically important infections, and monitoring the course of infection or the spread of a specific pathogen in a given population.

There are many areas of molecular diagnostics and many tools related to this field (Fig. 1.3). Not all molecular diagnostic tests are extremely expensive. Direct costs vary widely, depending on the complexity and sophistication of the test performed. Inexpensive molecular tests are generally kit based and use methods that require little instrumentation or technologist experience. DNA probe methods that detect *C. trachomatis* or *N. gonorrhoeae* are examples of low-cost molecular tests. The more complex molecular tests such as resistance genotyping often have high labor costs because they require experienced, well-trained technologists. Although the more sophisticated tests may require expensive equipment (e.g., DNA sequencer) and reagents, advances in automation and the production of less expensive reagents promise to decrease these costs as well as technician time.

In general, molecular tests for infectious diseases have been more readily accepted for reimbursement; however, reimbursement is often on a case-by-case basis and may be slow and cumbersome. FDA approval of a test improves the likelihood that it will be reimbursed but does not ensure that the amount reimbursed will equal the cost of performing the test.

Molecular screening programs for infectious diseases are developed to detect symptomatic and asymptomatic diseases in individuals and groups. Persons at high risk, such as immuno-compromised patients or those attending family planning or obstetrical clinics, are screened for CMV and *Chlamydia*, respectively. Likewise, all blood donors are screened for blood borne pathogens. The financial outcome of such testing is unknown. The cost must be balanced against the benefits of earlier diagnosis and treatment and societal issues such as disease epidemiology and population management.

One of the most highly touted benefits of molecular testing for infectious diseases is the promise of earlier detection of certain pathogens. The rapid detection of *M. tuberculosis* directly in clinical specimens by PCR or other amplification-based methods is quite likely to be cost-effective in the management of tuberculosis. Other examples of infectious diseases that are amenable to molecular diagnosis and for which management can be improved by this technology include HSV (herpes simplex virus) encephalitis, *Helicobacter pylori* infection, and neuroborreliosis caused by *Borrelia burgdorferi*. For HSV encephalitis, detection of HSV in cerebrospinal fluid (CSF) can direct specific therapy and eliminate other tests including brain biopsy. Likewise, detection of *H. pylori* in gastric fluid can direct therapy and obviate the need for endoscopy and biopsy. PCR detection of *B. burgdorferi* in CSF is helpful in differentiating neuroborreliosis from other chronic neurologic conditions and chronic fatigue syndrome.

Molecular tests may be used to predict disease response to specific antimicrobial therapy (Fig. 1.4, Chinnery et al., 1999). Detection of specific resistance genes (*mec A, van A*) or point mutations resulting in resistance has been proved to be efficacious in managing diseases. Molecular based viral load testing has become a standard practice for patients with chronic hepatitis and AIDS. Viral load testing and genotyping of HCV are useful in determining the use of expensive therapy such as interferon therapy, and can be used to justify decisions on the extent and duration of therapy. With AIDS, viral load determinations and resistance genotyping have been used to select among the various protease inhibitor drugs available for treatment, improving patient's response and decreasing incidence of opportunistic infections.

Pharmacogenomics is the use of molecular based tests to predict the response to specific therapies and to monitor the response of the disease to the agents administered (Pfaller, 2001). The best examples of pharmacogenomics in infectious diseases are the use of viral load and resistance genotyping to select and monitor antiviral therapy of AIDS and chronic hepatitis. This application improves disease outcome, shortens the period of stay at hospital, reduces adverse events and toxicity, and facilitates cost-effective therapy by avoiding unnecessary expensive drugs, optimizing doses and timing, and eliminating ineffective drugs.

Molecular strain typing of microorganisms is now well recognized as an essential component of a comprehensive infection control program that also involves the infection control department, the infectious disease division, and pharmacy. The sequences of 16S ribosomal RNA sequences can be used to study the evolutionary relationship between bacteria. This region is highly conserved. The sequence in these variable regions is species specific. In this approach PCR primers, complementary to flanking conserved sequences are used to amplify the variable regions. The product is then sequenced and sequence compared against the database of the 16S sequence to identify the bacteria it is derived from. This approach to identification of the bacteria has particular advantage with organisms that cannot be easily cultured in the laboratory, as the DNA is amplified by PCR rather than the organisms being amplified by growing in a culture.

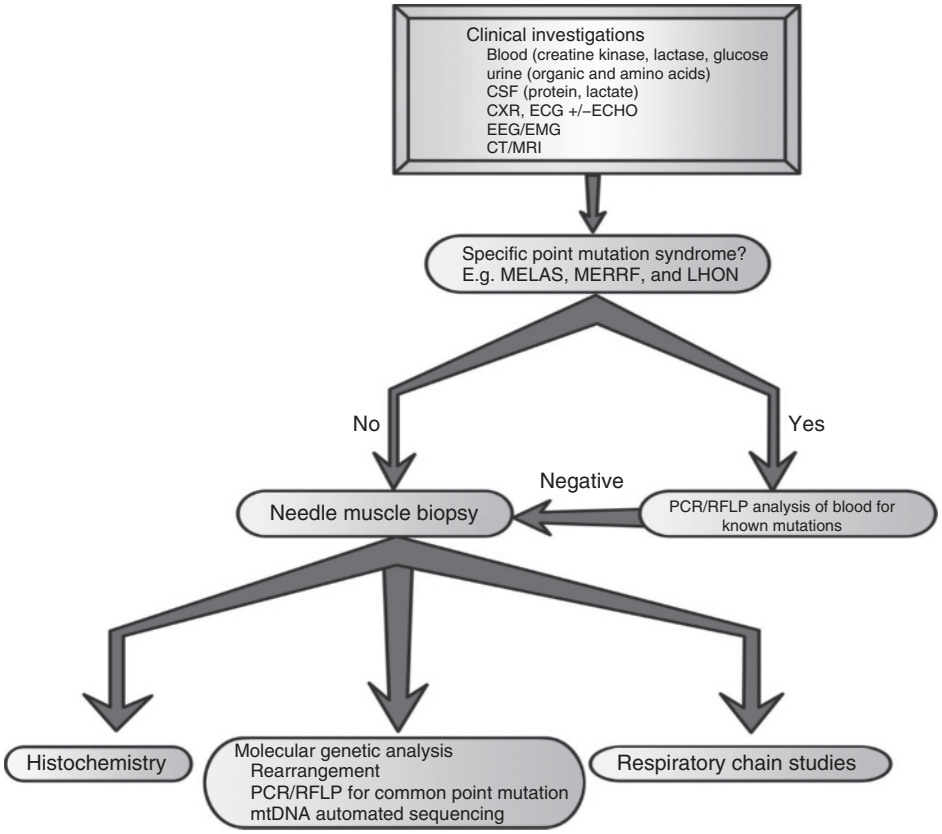

Figure 1.4. Molecular tests may be used to predict disease response to specific antimicrobial therapy.

Amplification of 16S genes by PCR can be very effective when combined with oligonucleotide hybridization probes or molecular beacon technology to identify bacteria in the mixture. A PCR reaction with a single set of primers complementary to conserved sequences will amplify species-specific sequence from a range of different bacteria in a mixture. These can then be probed with molecular beacons complementary to the individual species-specific sequences and labeled with different fluorophores. This makes it possible to identify more than one type of pathogenic bacterium in a mixture. By measuring the fluorescence levels in real time, it is also possible to make quantitative measurements and to detect the presence of a rare pathogen in the more abundant one.

One topical application of this technology is in devising tests to detect and identify bioterrorism agents. In the case of suspected bioterorrist attack, there is an urgent need for a robust and rapid assay for the selection of possible bioterrorist agents. A real-time PCR assay has been devised to simultaneously detect four bacteria having the potential to be used as bioterrorism agents by using a single set of PCR primers and four species-specific molecular beacons. In the case of bioterrorism accident, it would be vital to be able to identify the bacteria concerned. Technology has obvious applications in routine clinical laboratories where patient care could be improved by reducing the time taken.

1.2.4. Nanomedicine

The early genesis of the concept of nanomedicine sprang from the visionary idea that tiny nanorobots and related machines could be designed, manufactured, and introduced into the human body to perform cellular repairs at the molecular level. Today, it has branched out in hundreds of different directions, each of them embodying the key insight that the ability to structure materials and devices at the molecular scale can bring enormous immediate benefits in the research and practice of medicine. Nanomedicine is defined as the application of nanotechnologies including nanobiotechnologies in medicine. Evidently, dimensional parameters alone are insufficient to refer someone or other work to the field of nanotechnology (e.g., nanomedicine). Fundamental novelty of nanomedicine as a branch of knowledge and technology is exemplified by the developments in pharmacology and design of medicinal products that brought about new drugs (nanomedical/nanopharmaceutical). These products are multicomponent supramolecular compounds designed for a specific purpose whose intricate structure is intended not so much to impart new properties as to properly deliver the active ingredient to the biological target. Accordingly, nanomedicine should be regarded as the use of supramolecular complexes with a well-differentiated surface, manufactured by purposeful assembly of selected components for diagnostic and/or therapeutic application (Piotrovsky, 2010). Nanopharmaceuticals are defined as a big part of what nanomedicine is today. Nanopharmaceuticals can be developed either as drug delivery systems or as biologically active drug products (Table 1.1). Various types of nanomedicine are already in clinical use these days (Fig. 1.5).

DermaVir vaccine is a novel "pathogen-like" nanomedicine containing a plasmid DNA complexed with a polyethylenimine (pDNA/PEIm) that is mannobiosylated to target

TABLE 1.1. Various Types of Nanomedicine[a]

Type of Nanomedicine	Name of Nanomedicine	Use	Mode of Administration
Polymer as therapeutics	Copaxone	Multiple sclerosis	Parenteral injection
	Renagel	End-stage renal failure	Oral
	Emmelle	HIV/AIDS prevention	Topical
	Ampligen	Chronic fatigue syndrome	Parenteral injection
	Vivagel (Dendrimer)	HIV/AIDS prevention	Topical
	Macugen (PEG–aptamer)	Age-related macular degeneration	Topical
Polymer–protein conjugates	Adagen	SCID	Parenteral injection
	Zinostatin Stimaler (SMANCS)	Cancer	Local infusion
	Oncaspar	Cancer	Parenteral injection
	PEG–INTRON	Hepatitis C	Parenteral injection
	PEGASYS	Hepatitis C	Parenteral injection
	PEGvisomant	Acromegaly	Parenteral injection
	Neulasta	Cancer	Parenteral injection
	Cd870 (PEG–anti TNF–fab)	Crohn's disease Rheumatoid arthritis	Parenteral injection

[a] *Source*: From Ruth Ducan on nanopharmaceuticals.

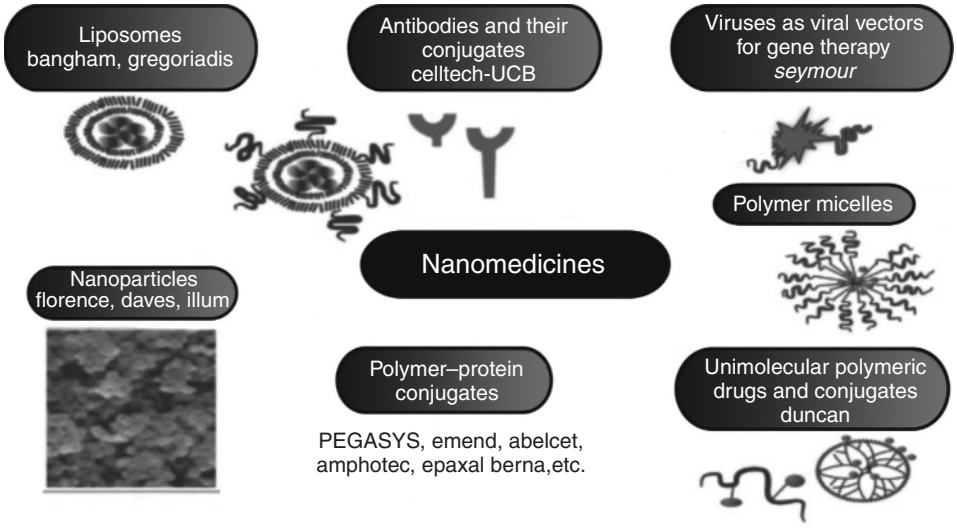

Figure 1.5. Various types of nanomedicines. (*See insert for color representation of the figure*).

antigen presenting cells and to induce immune responses. A commercially viable vaccine product was developed and the variability of raw materials and their relationship with the product's biological activity was investigated and found that the cGMP quality requirements are not sufficient to formulate the nanomedicine with optimal biological activity. The high cationic concentration of the pDNA favored the biological activity, but did not support the stability of the nanomedicine (Toke et al., 2010).

Nanomedicine also offers the prospect of powerful new tools for the treatment of human diseases and the augmentation of human biological systems (Table 1.2). They have been used as liposomes (3–100 nm) and nanoparticles (iron oxide, 5–50 nm) in clinical laboratories. To cure cancer, targeted drug delivery system is used using

TABLE 1.2. Application of Nanomedicine for Health

Application of Nanomedicine	Nanomaterial Name and Type	Pharmacological Function	Diseases
Nanomedicines in the clinic	Liposome (30–100 nm)	Targeted drug delivery	Cancer
	Nanoparticle (iron oxide, 5–50 nm)	Contrast agent for magnetic resonance imaging	Hepatic (liver)
Nanomedicines under development	Dendrimer (5–50 nm)	Contrast agent for magnetic resonance imaging	Cardiovascular phase III clinical trial
	Fullerenes (carbon buckyball 2–20 nm)	Antioxidant	Neurodegenerative, cardiovascular
	Nanoshells (gold-coated silica 60 nm)	Hyperthermia	Cancer preclinical

liposomes (Park, 2002). For hepatic diseases, nanoparticles are used as the contrast agent for generating resonance imaging (Thorek et al., 2006). Nanomedicine using dendrimers (Bharali et al., 2009), fullerenes (Partha and Conyers, 2009), and gold-coated nanoshells (Hirsch et al., 2003) is also under the developmental stage. In the cardiovascular phase III clinical trials, dendrimers are used as the contrast agent for magnetic resonance (Saha, 2009). Diamodoid-based medical nanorobotics may offer substantial improvements in capabilities over natural biological systems, exceeding even the improvements possible via tissue engineering and biotechnology. For example, the respirocytes, the artificial red blood cells comprise microscopic diamondoid pressure tanks that are operated at high atmospheric pressure and could carry >200 times respiratory gases than an equal volume of natural red blood cells.

Nanomedicine has been used to cure many diseases (Table 1.2). The clottocytes are artificial platelets that can stop human bleeding within ∼1 s of physical injury, but using only 0.01% the bloodstream concentration of natural platelets in other words, nanorobotic clottocytes would be ∼10,000 times more effective as clotting agents than an equal volume of natural platelets. In neurodegenerative diseases, carbon buckyballs (2–20 nm) are used as antioxidants. In preclinical cancer therapy during hyperthermia, gold-coated silica (60 nm) nanoshells are used.

Microbiovores constitute a potentially large class of medical nonorobots intended to be deployed in human patients for a wide variety of antimicrobial therapeutic purposes. They can also be useful in treating infections of the meninges or the CSF and respiratory diseases involving the presence of bacteria in the lungs or sputum, and can also digest bacterial biofilms. These handy nanorobots can quickly rid the blood of nonbacterial pathogens such as viruses (viremia), fungus cells (fungemia), or parasites (parasitemia).

A nanorobotic device that can safely provide quick and complete eradication of blood borne pathogens using relatively low doses of devices would be a welcome addition to the physician's therapeutic armamentarium Freitas, 2005. The ultimate tool of nanomedicine is the medical nanorobot—a robot to the size of a bacterium, composed of thousands of molecule-size mechanical parts perhaps resembling macroscale gears, bearings, and ratchets, possibly composed of a strong diamond-like material.

A nanorobot will need motors to make things move, and manipulator arms or mechanical legs for dexterity and mobility. It will have a power supply for energy, sensors to guide its actions, and an onboard computer to control its behavior. But, unlike a regular robot, a nanorobot will be very small. A nanorobot that would travel through the bloodstream must be smaller than the red cells—tiny enough to squeeze through even the narrowest capillaries in the human body.

Medical nanorobots could also be used to perform surgery on individual cells. In one proposed procedure, a cell repair nanorobot called a "chromallocyte," controlled by a physician, would extract all existing chromosomes from a diseased cell and insert fresh new ones in their place. This process is called *chromosome replacement therapy*. The replaced chromosomes are manufactured outside the patient's body using a desktop nanofactory optimized for organic molecules.

The patient's own individual genome serves as the blueprint to fabricate the new genetic material. Each chromallocyte is loaded with a single copy of a digitally corrected chromosome set. After injection, each device travels to its target tissue cell, enters the nucleus, replaces old worn out genes with new chromosome copies, then exits the cell and is removed from the body. If the patient chooses, inherited defective genes could be

replaced with nondefective base pair sequences, permanently curing any genetic disease and even permitting cancerous cells to be reprogrammed to a healthy state. Perhaps, most importantly, chromosome replacement therapy could correct the accumulating genetic damage and mutations that lead to aging in every one of our cells. At present, medical nanorobots are just theory. Nanorobots will have several advantages. Firstly, they can physically enter cells and scan the chemicals present inside. Secondly, they can have onboard computers that allow them to do calculations not available to immune cells. Thirdly, nanorobots can be programmed and deployed after a cancer is diagnosed, whereas the immune system is always guessing about whether a cancer exists. Given such molecular tools, a small device can be designed to identify and kill cancer cells (Saha, 2009).

The potential impact of medical nanorobotics is enormous. Rather than using drugs that act statistically and have unwanted side effects, we can deploy therapeutic nanomachines that act with digital precision, have no side effects, and can report exactly what they did back to the physician. Continuous medical monitoring by embedded nanorobotic systems will provide automatic collection of long-baseline physiologic data permitting detection of slowly developing chronic conditions that may take years or decades to develop, such as obesity, diabetes, calcium loss, or Alzheimer's. Nanorobot life cycle costs can be very low because nanorobots, unlike drugs and other consumable pharmaceutical agents are intended to be removed intact from the body after every use, then refurbished and recycled many times, possibly indefinitely. Even if the delivery of nanomedicine does not reduce total health-care expenditures—which it should—it will likely free up billions of dollars that are now spent on premiums for private and public health-insurance programs.

1.2.5. Personalized Medicine

The war against infectious agents has produced a powerful arsenal of therapeutics, but treatment with drugs can sometimes exacerbate the problem. The drug-resistant strains and the infectious agents that are least susceptible to drugs survive to infect again. They become the dominant variety in the microbe population, a present day example of natural selection in action. This leads to an ever-present concern that drugs can be rendered useless when the microbial world employs the survival of the fittest strategy of evolution. And, frequently used drugs contribute to their own demise by strengthening the resistance of many enemies.

Some engineering challenges are to develop better systems to rapidly assess a patient's genetic profile; another is collecting and managing massive amounts of data on individual patients; and yet another is the need to create inexpensive and rapid diagnostic devices such as gene chips and sensors able to detect minute amounts of chemicals in the blood. In addition, improved systems are necessary to find effective and safe drugs that can exploit the new knowledge of differences in individuals. The current "gold standard" for testing a drug's worth and safety is the randomized controlled clinical trial—a study that randomly assigns people to a new drug or to nothing at all, a placebo, to assess how the drug performs, but this approach essentially decides a drug's usefulness based on average results for the group of patients as a whole, not for the individual (Bottinger, 2007).

New methods are also needed for delivering personalized drugs quickly and efficiently to the site in the body where the disease is localized (Kalow, 2006). For instance,

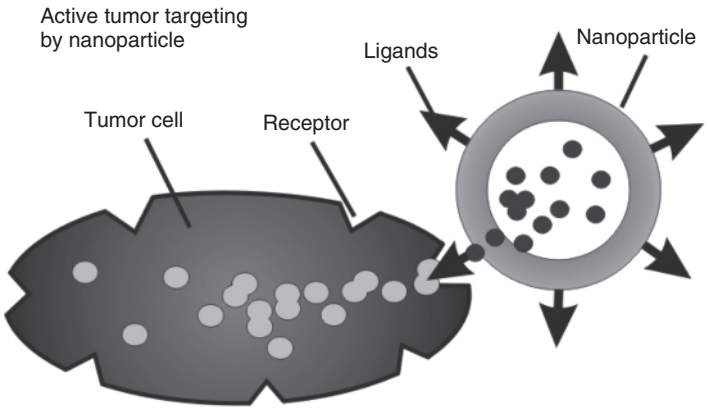

Figure 1.6. Drug delivery by nanoparticles.

researchers are exploring ways to engineer nanoparticles that are capable of delivering a drug to its target in the body while evading the body's natural immune response (Fig. 1.6). For example, when the drug-packed liposome is injected into the bloodstream, the amino acids on the nanoparticles attache to the proteins. The heat is pushed to the surface of the tumor and more of the drug is delivered to the tumor.

Such nanoparticles could be designed to be sensitive to the body's internal conditions, and, therefore, could, for example, release insulin only when the blood's glucose concentration is high. In a new field called *synthetic biology*, novel biomaterials are being engineered to replace or aid in the repair of damaged body tissues. Some are scaffolds that contain biological signals that attract stem cells and guide their growth into specific tissue types. Mastery of synthetic tissue engineering could make it possible to regenerate tissues and organs (Lutolf and Hubbell, 2005).

Ultimately, the personalization of medicine should have enormous benefits. It ought to make disease (and even the risk of disease) evident much earlier, when it can be treated more successfully or prevented altogether. It could reduce medical costs by identifying cases where expensive treatments are unnecessary or futile. It will reduce trial-and-error treatments and ensure that optimum doses of medicine are applied sooner.

More optimistically, personalized medicine could provide the path for curing cancer, by showing why some people contract cancer and others do not, or how some cancer patients survive when others do not. Thus, personalized medicine involves the use of laboratory-based molecular diagnostics and medical imaging to select suitable candidates for treatment with a particular drug(s), to rule out those patients who would suffer unacceptable side effects from the proposed drug treatment, and to monitor the health status of the patient postinitiation of therapy to assess therapeutic drug levels and the continuing efficacy of the agent in suppressing or curing the disease. Hence, personalized medicine is a new trend in drug development based on tailoring drugs to patients based on their individual genetic profiles.

Personalized-medicine-based pharmaceuticals avoid these issues by marketing to patients with specific genetic profiles that maximize both the safety and efficacy of the drug on each patient. Promising personalized-medicine-based pharmaceuticals in the pipeline have shown close to 100% efficacy in patients. Major pharmaceutical firms have responded to the growing emphasis on individualized therapy to improve drug efficacy

and safety with large investments in pharmacogenomics research (Mancinelli et al., 2000); Table 1.3). Examples of drugs in the market that have used genetic markers to achieve improved safety and efficacy in patients include Gleevec [Novartis AG (NVS)] and Herceptin [Genentech (DNA)] (http://www.wikinvest.com). Of course, a transition to personalized medicine is not without its social and ethical problems.

Even if the technical challenges can be met, there are issues of privacy when unveiling a person's unique biological profile, and it is likely that there will still be masses of people throughout the world unable to access its benefits deep into the century.

The drug resistance problem is not limited to bacteria and antibiotics. Antiviral drugs for fighting diseases such as AIDS and influenza face similar problems from emerging strains of resistant viruses (Wright and Sutherland, 2007). In fact, understanding the development of resistance in viruses is especially critical for designing strategies to prevent pandemics. The use of any antimicrobial drug must be weighed against its contribution to speeding up the appearance of resistant strains.

The engineering challenges for enabling drug discovery mirror those needed to enable personalized medicine development: more effective tools and techniques. This helps in rapid analysis and diagnosis so that a variety of drugs can be quickly screened and proper treatments can be promptly applied (West et al., 2006). Current drugs are often prescribed incorrectly or unnecessarily, promoting the development of resistance without real medical benefit. Quicker, more precise diagnosis may lead to more targeted and effective therapies. Antibiotics that attack a wide range of bacteria have typically been sought, because doctors could not always be sure of the precise bacterium causing an infection. Instruments that can determine the real culprit right away could lead to the use of more narrowly targeted drugs, reducing the risk of promoting resistance. Developing organism-specific antibiotics could become one of the century's most important biomedical engineering challenges. Personalized medicine will reshape pharmaceutical research and development and the calculation of cost-effectiveness by health services. The previous business model was based on so-called blockbuster drugs, intended for general use in the population and generating annual global profits in excess of $1 billion. Profits from blockbuster drugs offset the expenses of regulatory approval and investment in research and development (http://www.parliament.uk/documents/post/postpn329.pdf).

This could be especially challenging in the case of biological agents specifically designed to be weapons. A system must be in place to rapidly analyze their methods of attacking the body and quickly produce an appropriate medicine. In the case of a virus, small molecules might be engineered to turn off the microbe's reproductive machinery. Instructions for making proteins are stored by genes in DNA. Another biochemical molecule, called *messenger RNA*, copies those instructions and carries them to the cell's protein factories (Dietel and Sers, 2006). Sometimes other small RNA molecules can attach to the messenger RNA and deactivate it, thereby preventing protein production by blocking the messenger, a process known as *RNA interference*. Viruses can be blocked by small RNAs in the same manner, if the proper small RNAs can be produced to attach to and deactivate the molecules that reproduce the virus. The key is to decipher rapidly the sequence of chemicals comprising the virus so that effective small RNA molecules can be designed and deployed.

TABLE 1.3. Selected Pharmaceutical Companies Focusing on Genomics and Pharmacogenomics

Company	Web Address	Focus
ACLARA BioSciences, Inc.	http://www.aclara.com	Lab card microfluidic technology
Aeiveos Sciences Group, LLC	www.aeiveos.com	Aging-related genes and gene responses
Affymetrix, Inc.	http://www.affymetrix.com	GeneChip microarray technology
Aurora Bioscience Corp	http://www.aurorabio.com	Genomic and drug screening technology
Axys Pharmaceuticals Inc./PPGx	http://www.axyspharm.com	Pharmacogenomics (with PDD Inc.)
Caliper Technologies Corp	http://www.clipertech.com	Microfluidic Lab Chip, SNP scanning (with Agilent)
Celera Genomics	http://www.celera.com	Human Genome sequencing and SNP scanning
Cellomics, Inc.	http://www.cellomics.com/	Pharmacocellomics, cellular bioinformatics
Curagen Corp	http://www.curagen.com/	SNP scanning; gene expression and drug response
Epidauros	http://www.epidauros.com/	Pharmacogenomics in drug discovery and therapy
Exelixis, Inc.	http://www.exelixis.com	Model systems for drug discovery
Eurona Medical, AB	http://www.eurona.com/	Drug responses and genetic profiling
Gemini Research, Ltd	http://www.gemini-research.co.uk/	Gene discovery; dizygotic twin studies
Genaissance Pharmaceuticals, Inc.	http://www.genaissance.com	Genetic polymorphism in cancer, vascular lesions
Gene Logic, Inc.	http://www.genelogic.com	Gene expression databases
Genome Therapeutics Corp	http://www.crik.com/	Human high-resolution polymorphism database
Genometrix, Inc.	http://www.genometrix.com	DNA microarrays
Genomic Solutions, Inc.	http://www.genomesolutions.com/	Genomics
Genset, SA	http://www.genset.fr	High-density biallelic maps; SNP identification
Hexagen Pic	http://www.hexagen.co.uk/	Single-strand conformational assay of polymorphisms
Hyseq, Inc.	http://www.hyseq.com	Genomic methods for therapeutic discovery
Incyte Pharmaceuticals, Inc.	http://www.incyte.com	Bioinformatics, SNP scanning, functional genomics
Kiva Genetics	http://www.kivagen.com	Pharmacogenetic testing services

(*continued*)

TABLE 1.3. (*Continued*)

Company	Web Address	Focus
Lion Bioscience, AG	http://www.lion-ag.de/	Bioinformatics, drug targets from gene expression
Lynx Therapeutics	http://www.lynxgen.com	Microbead-based DNA/SNP scanning
Microcide Pharmaceuticals	http://www.microcide.com/	Microbial genomics and antibiotics
Mitokor, Inc.	http://www.mitokor.com/	Mitochondrial genome analysis
Nova Molecular, Inc.	http://www.cns-hts.com/	CNS disease profiling
Millennium Predictive Medicine	http://www.mlnm.com/subsid/mpmx.html	Pharmacogenomics, predicting disease and therapy
Orchid Biocomputer, Inc.	http://www.orchidbio.com	Microfluidic devices and pharmacogenetic testing
PE Biosystems, Inc.	http://www.pebio.com	Genomics, drug discovery
PPGx	http://www.ppgx.com	Pharmacogenetic testing services
Protogene Laboratories	http://www.protogene.com	DNA microarray development
Rigel, Inc.	http://www.rigelinc.com/	Identification of genetic drug targets
Rosetta Inpharmatics	http://www.rii.com/	Oligonucleotide array studies
Third Wave Technologies, Inc.	http://www.twt.com/	SNP scanning, pharmacogenomics
Transgenomic, Inc.	http://www.transgenomic.com/	Discovery of genetic variations
Variagenics, Inc.	http://www.variagenics.com/	Cancer therapeutics based on loss of heterozygosity

Traditional vaccines have demonstrated the ability to prevent diseases, and even eradicate some such as smallpox. It may be possible to design vaccines to treat diseases as well. Personalized vaccines might be envisioned for either use. But, more effective and reliable manufacturing methods are needed for vaccines, especially when responding to a need for mass immunization in the face of a pandemic (Heymann, 2006). A healthy future for the world's population will depend on engineering new strategies to overcome multiple drug resistances (Gerard and Sutherland, 2007). One major challenge in this endeavor will be to understand more fully how drug resistance comes about, how it evolves, and how it spreads. Furthermore, the system for finding and developing new drugs must itself evolve, and entirely novel approaches to fighting pathogens may also be needed (Kalow, 2006).

Drug resistance is nothing new. The traditional approach to this problem is still potentially useful in expanding the search for new antibiotics. Historically, many drugs to fight disease-producing microbes have been found as naturally occurring chemicals in soil bacteria, which is still a source of promising candidate (Lesko, 2007). Even more

drug candidates, though, may be available from microbes in more specialized ecological niches or from plants or from bacteria living in remote or harsh environments, namely, deep lakes and oceans.

1.2.6. Biowarfare

Biological warfare agents are a group of pathogens and toxins of biological origin that can be potentially misused for military or criminal purposes (Pohanka and Kuca, 2010). In early December 2002, the National Security Council learned of a smallpox outbreak in Oklahoma. Twenty cases were confirmed by the Centres for Disease Control and Prevention (CDC), with 14 more suspected. There were 16 more reported cases in Georgia and Pennsylvania. Federal and State authorities quickly informed the public and implemented a vaccine distribution policy to those people most at risk of being exposed to the smallpox virus (http://learn.genetics.utah.edu). Three days before the Christmas holiday and 13 days after the initial outbreak, a total of 16,000 smallpox cases were reported in 25 states, and 1000 people were dead. Ten other countries reported cases of smallpox, likely to be caused by visitors from the United States. Canada and Mexico closed their borders to the United States. Vaccine supplies were depleted, and health officials predicted that by February, there will be three million cases of smallpox, leading to as many as one million deaths. The above scenario was, in fact, a game.

In their natural state, bacteria, viruses, and fungi can make pretty good biological weapons. If genetic engineering is used, more harmful agents can emerge. During the Cold War, several offensive biowarfare programs were run to develop the so-called Super Bugs. One such program, Project Bonfire, worked to create bacteria that were resistant to about 10 varieties of antibiotics. This was done by identifying and cutting out genes that conferred antibiotic resistance in many different strains of bacteria. By pasting these genes into the DNA of the anthrax bacterium, the Project Bonfire created a strain of anthrax that resisted any existing cure, making it impossible to treat (Fig. 1.7). The Hunter Program was another biological warfare research program that focused on combining whole genomes of different viruses to produce completely new hybrid viruses (Fig. 1.8). These artificial viruses could cause unpredictable symptoms that have no known treatment. In an innovative twist, the Hunter Program also created bacterial strains that carried pathogenic viruses inside them. These strains would be double trouble: a person who contracted the bacterial disease would likely be treated with an antibiotic, which would stop the infection by disrupting the bacterial cells. This would release the virus, resulting in an outbreak of viral disease. Such a scenario would confuse medical personnel, making treatment very difficult.

It is not known whether the biological agents were ever actually used to infect people. At the same time, there was mounting fear over the offensive biological warfare agenda, focusing on the difficult task of delivering biological weapons such as anthrax to a population. In experiments conducted at sea or at the desert facility, large populations of animals such as guinea pigs, monkeys, and sheep were exposed to these deadly agents. Even more unsettling was the research to test the dispersal of bacterial spores on human populations; harmless bacterial spores that mimicked anthrax in size and shape were covertly dispersed in the ventilation system, over the bay and in the subways. Results from these experiments revealed not only the difficulty of delivering anthrax to a populace, but also the deadly consequences of a successful distribution (http://learn.genetics.utah.edu).

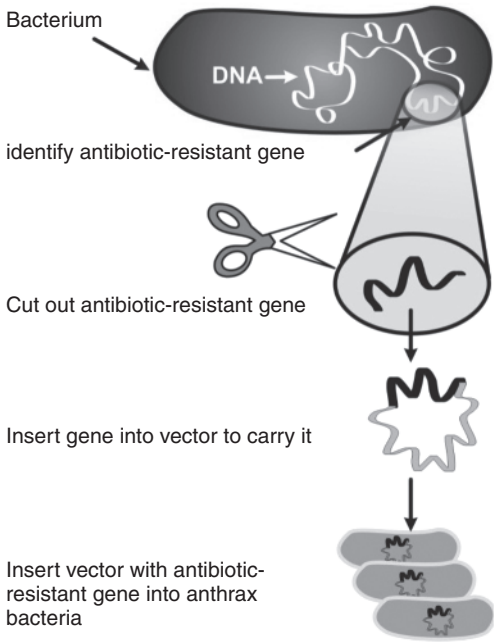

Figure 1.7. Project Bonfire: creation of antibiotic-resistant bacteria.

The existence of these experiments was unknown to the public until the 1970s, and the actual results were not revealed until 1999, when some of the data was declassified by the Department of Defence. Advances in genetics may soon make possible the development of ethnic bioweapons that target specific ethnic or racial groups based on genetic markers (Appel, 2009). The tools for specific defense against bioweapons consist of vaccines against both viruses and bacteria, and of antibiotics and drugs against bacteria.

Vaccines and antimicrobials are of limited usefulness because of the large number of possible microbes that can be used for weapons, because of antimicrobial resistance to drugs and antibiotics, and because of limitations in technical feasibility for developing vaccines and antibacterials against certain agents. Induction of nonspecific innate immunity by immunostimulatory vaccines (at one time licensed) needs to be explored for possible immunoprophylactic–therapeutic activity when administered immediately following exposure to bioweapon pathogens.

Research into the offensive use of biological weapons has been carried out all over the world (Alibek, 2004; Guillemin, 2005; Karwa et al., 2005; Guillemin, 2006). The agents of biowarfare under category A are the high priority agents that include organisms that pose a high risk to national security because they can be disseminated/transmitted from person to person. The agents for biowarfare are botulism, small pox, marburg, plaque, and turaremia. The investigation on the use of *Bacillus anthracis* (anthrax), botulinum toxin (botulism), *Yersinia pestis* (plague), *Francisella tularensis* (tularemia), *Coxiella burnetii* (Q fever), Venezuelan equine encephalitis virus, *Brucella suis* (brucellosis), and Staphylococcal enterotoxin B has already been made.

This treaty prohibits the stockpiling of biological agents for offensive military purposes, and also forbids research into offensive use of biological agents. From 1975

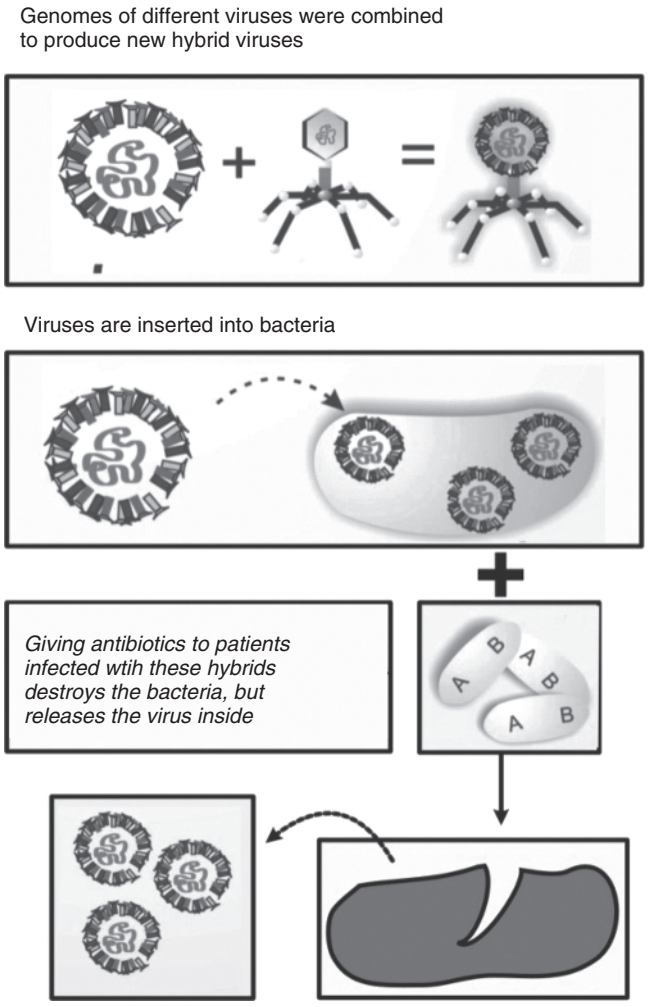

Figure 1.8. Hunter Program: creation of hybrid viruses.

to 1983, Soviet-backed forces in Laos, Cambodia, and Afghanistan allegedly used tricothecene mycotoxins (T-2 toxins) in what was called "Yellow Rain" (Seeley et al., 1985). After being exposed, people and animals became disoriented and ill, and a small percentage of those stricken died. The use of T-2 toxins has been denied and the presence of the yellow spots was reported as being caused by defecating bees. Various molecular methods of detection of the agents, such as PCR, hybridization, and strain typing, were performed on the basis of bacterial total cell protein profiles, random amplified polymorphic DNA (RAPD), and ribotyping, as well as of plasmid and DNA microrestriction analyses for the identification of agents of bioterrorism and biowarfare.

Various commercial tests utilizing biochemical, immunological, nucleic acid, and bioluminescence procedures are currently available to identify biological threat agents.

Newer tests have also been developed to identify such agents using aptamers, biochips, evanescent wave biosensors, cantilevers, living cells, and other innovative technologies (Lim et al., 2005; Carter and Cary, 2007; Garnier et al., 2009).

The pathogenic *Burkholderia mallei* and *Burkholderia pseudomallei* are the causative agents of glanders and melioidosis, respectively, in humans and animals, and are regarded as potential agents of bioterrorism. The existing bacteriological and immunological methods of identification of *B. mallei* and *B. pseudomallei* are not efficient enough for the rapid diagnosis and typing of strains (Antonov and Iliukhin, 2005). Schmoock et al. (2009) reported a DNA-microarray-based detection and identification of *Burkholderia mallei, Burkholderia pseudomallei*, and *Burkholderia* spp.

1.3. THE MICROBIAL WORLD

For most of its history, life on earth consisted solely of microscopic life-forms, and microbial life still dominates earth in many aspects (Table 1.4; (di Castri and Younes, 1990; Corliss, 1991; Hawksworth, 1991). The estimated 5×10^{30} prokaryotic cells inhabiting our planet sequester some 350–550 Pg (1 Pg = 1015 g) of carbon, 85–130 Pg of nitrogen, and 9–14 Pg of phosphorus making them the largest reservoir of those nutrients on earth (Whitman et al., 1998). Bacteria and archaea live in all environments capable of sustaining other life and in many cases are the sole inhabitants of extreme environments: from deep sea vents to rocks found in boreholes 6 km beneath the earth's surface. Bacteria, archaea, and microeukaryotes dominate earth's habitats, compound recycling, nutrient sequestration, and, according to some estimates, biomass. Microbes are not only ubiquitous, they are essential to all life, as they are the primary source for nutrients and the primary recyclers of dead matter back to available organic form. Along with all other animals and plants, the human condition is profoundly affected by microbes, from the scourges of human, farm animal, and crop pandemics, to the benefits in agriculture, food industry, and medicine, to name a few (Wooley et al., 2010).

Microbes are believed to be the common ancestors of all organisms. They not only grow virtually everywhere but also are present in abundance. In contrast to the relatively small number of humans (6×10^9), populations of terrestrial and marine bacteria are immense, 5×10^{30} and 1.2×10^{29}, respectively (Whitman et al., 1998). In fact, the human body contains 10 times more bacterial cells than human cells. Microbes carry

TABLE 1.4. Conservatively Estimated Total Microorganism Species in the World

Group	Known Species	Estimated Total Species	Percentage of Known Species
Viruses	5,000	1,30,000	4
Bacteria	4,760	40,000	12
Algae	40,000	60,000	67
Fungi and lichen	69,000	15,00,000	5
Protozoa	40,000	1,00,000	31
Total	1,57,000	18,20,000	9

out innumerable transformations of matter that are essential to life and thus have an enormous effect on climate and the geosphere.

We humans have more bacterial cells (10^{14}) inhabiting our body than our own cells (10^{13}) (Salvage, 1977; Berg, 1996). Scientists realized in the early 1990s that only a small fraction (1%) of microbes in natural communities was known and these communities became the focus of many studies. The sequencing of genomes of our own microbes is necessary (Wooley et al., 2010). Several years later, however, it is still shocking to realize the depth of our ignorance, which is well illustrated by the story of the SAR11 clade. When the technique of ribotyping (cloning and sequencing 16S rRNA genes) was first used to survey natural ecosystems, SAR11 was one of the first groups of novel microbes to be discovered (Giovannoni et al., 1990). We now know that the highest percentage of bacterial 16S ribosomal genes present in all oceanic and coastal waters is from members of the SAR11 clade, making this group "one of the most successful clades of organisms on the planet" (Giovannoni et al., 2005).

The basis for classification by ribotyping is the sequence of the 16S ribosomal RNA gene in prokaryotes and the 18S gene in eukaryotes. The 16S and 18S rRNA genes were selected for classification and identification of microbes because these genes are universal and essential; all living organisms must synthesize proteins to survive (Woese and Fox, 1977). These genes are also well suited for this purpose because they contain both conserved and variable regions, as is evident in the nucleotide sequence of the 16S gene.

Microbes are essential to human survival, performing tasks that the human body on its own cannot. This means the human body is more of an amalgam of different life-forms rather than being alone organism. It is believed that microbes coevolved with humans, with each supporting the other's survival. Interestingly, other research suggests people are not born with microbes, but pick them up from their environment. We know that microbial flora plays a significant role in human health and disease. There is also a shift in thinking about the role of microbes. While it was thought that individual microbes performed specific tasks on their own, there is now thought that some of them may even work together. Evidence suggests that signals are transmitted between some groups of microbes (http://www.accessexcellence.org). In addition, microbial flora populations can vary between individuals (Fig. 1.9).

1.3.1. Classification System

Whittaker based his kingdom groupings on the three main modes of nutrition in natural communities: absorption, ingestion, and autotrophy. He also credited the evolutionary sequence of unicellular to multicellular with central importance to his classification scheme. Utilizing these criteria as the basis for classification, Whittaker returned the bacteria to kingdom Protista (also based on their unicellular nature) and placed all algae (green, brown, and red) into kingdom Plantae. The protozoa were reassigned by Whittaker (1959) to his kingdom Protista. Whittaker's primary phyletic interest overall was, however, in establishing a separate kingdom to contain macroscopic fungi. In particular, Whittaker observed the absorptive role of the fungi in the natural environment. He rejected the common belief that the superficial resemblance of fungi to plants, with their nonmotile habit and cell walls, made them true plants. Whittaker did not believe that the fungi were derived from algae, but rather he thought they evolved from "colorless, flagellated protist ancestors." In 1969, Whittaker published a revision of his four-kingdom system to expand it to five kingdoms, now including a separate bacterial

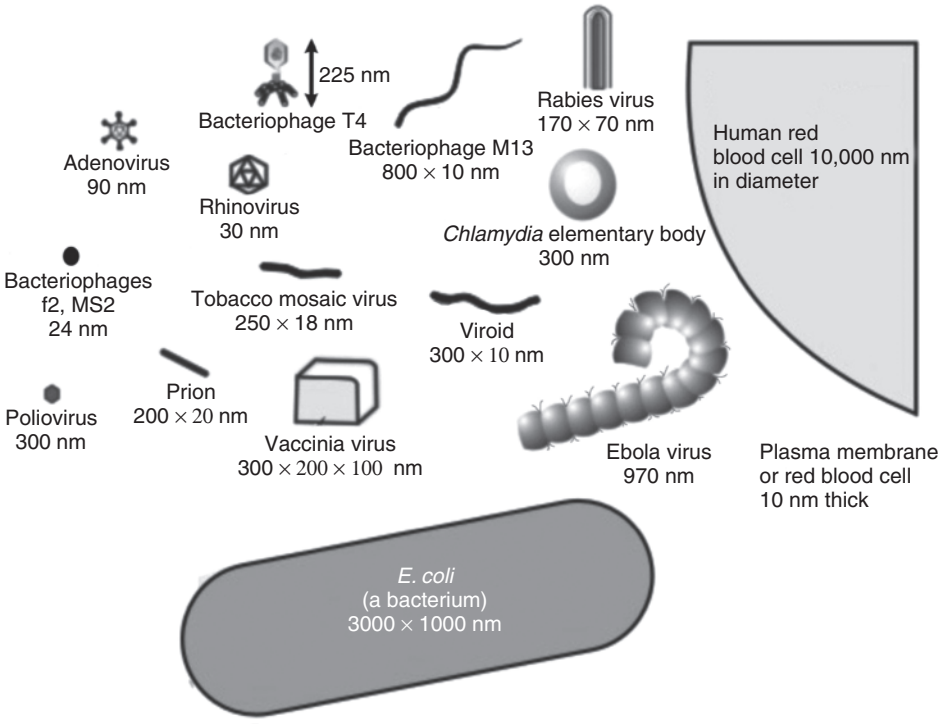

Figure 1.9. Morphological diversity in microbes.

kingdom named *Monera* in recognition of the fundamental division of life as "prokaryotic" versus "eukaryotic." Whittaker noted that this concept was now more evident. Otherwise, Whittaker's reasoning remained the same for retaining the other four kingdoms in this five-kingdom system (Fig. 1.10). He reasserted his ecological model as well as the belief that inclusion of multicellular organisms into kingdom Protista would make the five-kingdom system an evolutionarily unnatural, heterogeneous grouping.

1.3.2. Viruses, Viroids, and Prions

In order to be alive, an organism must be composed of one or more cells. Viroids and prions are not living things and so are termed as agents. Many infectious agents consist of only a few of the molecules typically found in cells. Viruses consist of a piece of nucleic acid surrounded by a protective protein coat. They come in a variety of shapes, conferred by the shape of the coat. Viruses share with all organisms and agents the need to reproduce copies of themselves; otherwise they would not exist in nature. Viruses can only multiply inside living host cells, whose machinery and nutrients they must borrow for reproduction. Otherwise inside the host, they are inactive. One way of classifying viruses is to group them broadly into animal viruses, plant viruses.

A more fundamental classification separates them into two categories: DNA and RNA viruses (Dimijian, 2000; Table 1.5). Thus, viruses may be considered obligate intracellular parasites. All forms of life including members of the bacteria, archae, and eucarya can be infected by viruses. Although viruses frequently kill the cells in which they multiply,

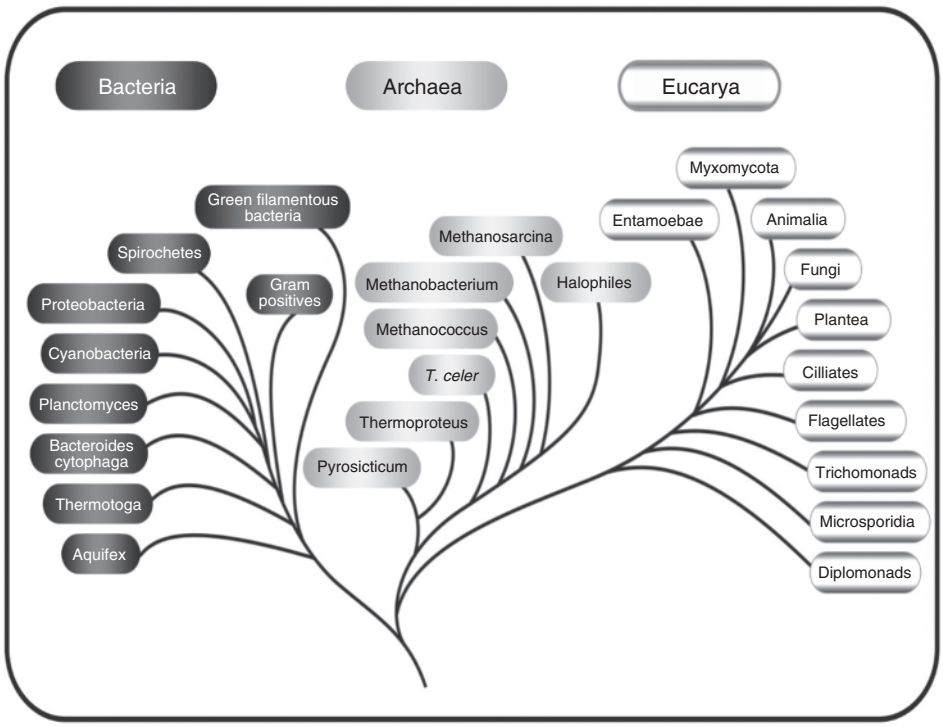

Figure 1.10. Phylogenetic tree of life.

TABLE 1.5. Classification of Viruses

Type	
DNA viruses	Herpes viruses
	Adenoviruses
	Hepatitis viruses
	Bacteriophages
RNA viruses	Retro viruses
	Influenza viruses
	Hepatitis virus A, C, and D
	Hemorrhagic fever viruses (dengue viruses, hantaviruses, Ebola virus)

some viruses exist harmoniously within the host cell without causing obvious ill effects. Some even cause clinical and special effects to human beings (Table 1.6).

Viriods define a group of pathogens that are also much smaller and simpler than viruses, consisting of a single short piece of single-stranded RNAthat varies from 246 to 375 nucleotides without a protective coat. Hence, they are resistant to proteases. A single viriod RNA molecule is capable of infecting a cell. The viriod RNA is circular and is resistant to digestion by nucleases. They are smaller than viruses and like viruses; they can reproduce only inside cells. Viriods replicate autonomously within susceptible cells. No other virions or viriods are required for their replication. Viroids

TABLE 1.6. Families of Viruses that Affect Humans

Characteristics/ Dimensions	Viral Family	Important Genera	Clinical or Special Features
Single-stranded DNA nonenveloped 18–25 nm	Parvoviridae 	Human parvovirus B19	Fifth disease; anemia in immuno-compromised patients
Double-stranded DNA nonenveloped 70–90 nm	Adenoviridae 	Mastadenovirus	Medium-sized viruses that cause various respiratory infections in humans; some cause tumors in animals
40–57 nm	Papovariridae 	Papillomavirus (human wort virus) Polyomavirus	Small viruses that induce tumors; the human wort virus (papilloma) and certain viruses that produce cancer in animals (polyoma and simian) belong to this family
Double-stranded DNA-enveloped 200–350 nm	Poxviridae 	Orthopoxvirus (vaccinia and small pox viruses) Molluscipoxvirus	Very large, complex, brick-shaped viruses that cause diseases such as smallpox (variola), molluscum contagiosum (wortlike skin lesion), and cowpox
150–200 nm	Herpesviridae 	Simplexvirus (HHV-1 & 2) Varicellovirus (HHV-3) Lymphocryptovirus (HHV-4) Cytomegalovirus (HHV-5) Roseolovirus (HHV-6) HHV-7 Kaposi's sarcoma (HHV-8)	Medium-sized viruses that cause various human diseases such as fever blisters, chickenpox, shingles, and infectious mononucleosis; implicated in a type of human cancer called Burkit's lymphoma
42 nm	Hepadnoviridae 	Hepadnavirus (hepatitis B virus)	After protein synthesis, hepatitis B virus uses reverse transcriptase to produce its DNA from mRNA; causes hepatitis B and liver tumors

cause a number of plant disease, and some scientists speculate that they cause diseases in humans.

Prions are very unusual agents that are responsible for at least six neurodegenerative diseases in humans and animals, they are always fatal. They are proteinaceous infectious agents that apparently contain only protein and no nucleic acids. Although it is unlikely, it is possible that another agent that is very difficult to isolate might also be involved in causing the neurodegenerative disease. In all these infections, brain function degenerates as neurons die and brain tissue develop sponge-like holes. Thus, the term *transmissible spongiform encephalopathies* (*TSEs*) has been given to all of the diseases. Prions have many properties of viruses, but their evolutionary relationship to viruses is unclear. Both viruses and prions are obligate intracellular parasites, but prions are smaller than the smallest viruses, and unlike viruses or any other replicating agents contain no nucleic acids. Prions are not inactivated by UV light or nucleases, but are inactivated by chemicals that denature proteins as well as heat.

One of the most intriguing questions regarding prions is how they replicate if they do not contain any nucleic acids. The prion replicates by converting the normal host protein into a prion protein, thereby creating more prion protein molecules (Fig. 1.11). Thus, the prion protein is infectious because it catalyzes the conversion of normal protein into a prion protein by changing the folding properties of the protein.

In most cases, the prion disease is only transmitted to members of the same species, because the amino acid sequence of different prion protein in different species differs from one another. However, the barrier to prion transmission between species also depends on the strain of the prion. It is now clear that the strain of the prion that causes mad cow disease in England has killed more than 100 people by causing a disease very similar to Creutzfeldt–Jakob disease; presumably, these people ate beef of infected animals. Thus so far no deaths have been attributed to eating sheep infected with the scrapie agent or deer and eek infected with the prion causing chronic wasting disease. However, because

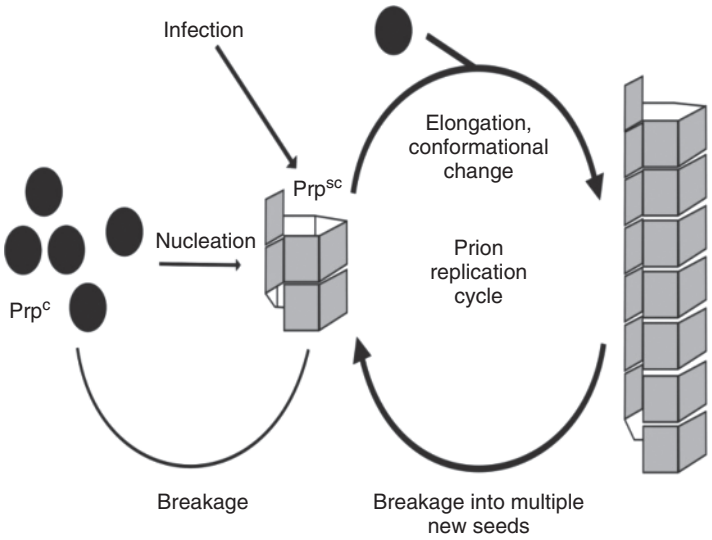

Figure 1.11. Replication of prions.

the incubation period extends over many years, the possibility that cross infection can occur in this situation also has not been ruled away completely.

1.3.2.1. Bacterial Viruses

A virus, injects its genome into a host bacterium, initiating production of new viruses and viral DNA. Bacterial viruses or bacteriophages are viruses that infect bacteria. It has a 48 kb double-stranded DNA genome packaged into an icosahedral head. Its genome is linear. An example is λ phage. The λ DNA when enters *E.coli* the ends of the linear genome join together to produce a circular genome. On infection, λ can either follow a lysogenic or lytic pathway. During the lysogenic pathway, the viral genome is integrated into the host genome and then replicated as part of the host genome; no expression of the viral structural proteins occurs in this case. If λ enters the lytic pathway, proteins required for the formation of the progeny virions are made and the viral genome is replicated and progeny virions are formed. The cell lyses and about 100 new virions are released. The virus contains a very compact genome coding for the 46 genes. λ has been exploited as a vector for DNA cloning predominantly with which it inserts its DNA into *E. coli*. They are perhaps the best understood viruses, yet at the same time, their structure can be extraordinarily complex (Fig. 1.12). They were originally distinguished by their smallness (hence, they were described as "filterable" because of their ability to pass through bacteria retaining filters) and their inability to replicate outside of a living host cell. Because these properties are shared by certain bacteria (rickettsiae, chlamydiae), viruses are further characterized by their simple organization and their unique mode of replication. A virus consists of genetic material, which may be either DNA or RNA, and is surrounded by a protein coat, and, in some viruses, by a membranous envelope.

Unlike cellular organisms, viruses do not contain all the biochemical mechanisms for their own replication; viruses replicate by using the biochemical mechanisms of a host cell to synthesize and assemble their separate components. When a complete virus

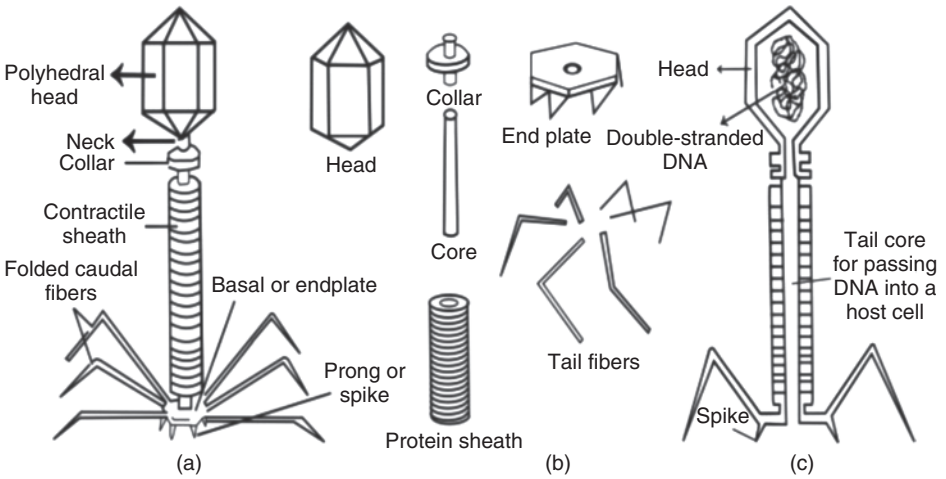

Figure 1.12. Bacteriophage: (a) T_4 phage, (b) isolated parts of the phage, (c) diagrammatic section showing the location of dsDNA in the head.

particle (virion) comes in contact with a host cell, the viral nucleic acid and, in some viruses, a few enzymes are introduced into the host cell.

Viruses vary in their stability, some such as poxviruses, parvoviruses, and rotaviruses are very stable and survive well outside the body while others, particularly those viruses such as herpes virus and influenza virus that are enveloped, do not survive well and therefore usually require close contact for transmission and are readily destroyed by disinfectants, particularly those with a detergent action. Some viruses produce acute disease while others, sometimes referred to as *slow viruses* such as retroviruses and lentiviruses and the scrapie agent, produce diseases that progress often to death over many years. Viruses in several families are transmitted by arthropod vectors. Bacteriophages continue to play a key role in bacterial genetics and molecular biology. Phage can confer key phenotypes on their host, for example, converting a nonpathogenic strain into a pathogen, and they play a key role in regulating bacterial populations in all sorts of environment (Fig. 1.13).

The use of bacteriophages was important in discovering that DNA in viruses can reproduce through two mechanisms: the lytic cycle and the lysogenic cycle. When viruses reproduce by the lytic cycle, they break open, or lyse, their host cells, resulting in the destruction of the host. In the lysogenic cycle, the phage's DNA (viral DNA) recombines with the bacterial chromosome. Once it has inserted itself, it is known as a prophage. A host cell that carries a prophage has the potential to lyse, thus it is called a lysogenic cell.

The phage–bacterium relationship varies enormously. From the simple predator–prey model to a complex, almost symbiotic relationship that promotes the survival and evolutionary success of both. While infection of bacteria used in the fermentation industry

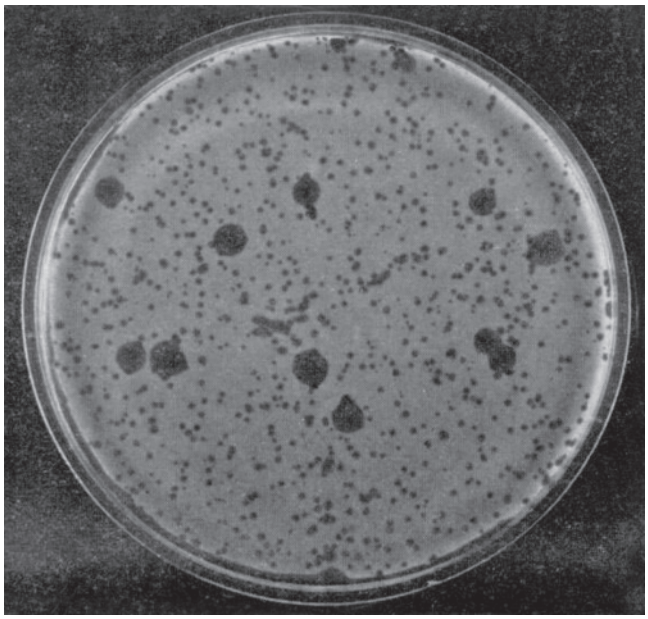

Figure 1.13. Plaques of different sizes caused by at least two bacteriophages on a carpet of actively multiplying bacteria.

can be very problematic and result in financial losses, in other scenarios phage infection of bacteria can be exploited for industrial and/or medical applications. In fact, interest in phage and phage gene products as potential therapeutic agents is increasing rapidly and is likely to have a profound impact on the pharmaceutical industry and biotechnology, in general, over the coming years. One potential application is the use of phage to combat the growing menace of antibiotic-resistant infections.

1.3.3. Bacteria

Bacteria are most widely distributed; they have simple morphology, are small in size, are the most difficult to classify and are the most hard to identify. It is even difficult to provide a descriptive definition of what a bacterial organism is because of considerable diversity in the group. About the only generalization that can be made for the entire group is that they are prokaryotic and some are photosynthetic. A few of them utilize a pigment that is chemically different from the chlorophyll *a*. It is called bacteriochlorophyll. Probably, the simplest definition that one can construct from these facts is bacteria are prokaryons without chlorophyll *a*.

Since they are prokaryons, bacteria share the kingdom Monera with the cyanobacteria. Although bacteria are generally smaller than cyanobacteria, some of the cyanobacteria range in size as that of bacteria. Most bacteria are only 0.5–2.0 μm in diameter. The various shapes of bacteria encountered are mainly grouped into three types: rod, spherical, and helical or curved. Rod-shaped bacteria may vary considerably in length, may have square, round, or pointed ends, and may be motile or nonmotile.

The spherical- or coccus-shaped bacteria may occur as a single bacterium, or in pairs, in tetrads, in chains, and in irregular masses. The helical and curved bacteria exist as slender spirochetes, spirullum, and bent rods (vibrios) (Fig. 1.14).

1.3.3.1. Cyanobacteria

Cyanobacteria get their name from the bluish pigment phycocyanin, which they use to capture light for photosynthesis. The cyanobacteria, blue-green bacteria, constitute subkingdom I of the kingdom Monera. These microorganisms were formerly referred to as *algae*. Cyanobacteria can be found in almost every conceivable environment, from oceans to freshwater to bare rock to soil. They can occur as planktonic cells or form phototropic biofilms in freshwater and marine environments, they occur in damp soil, or even temporarily moistened rocks in deserts. The ability of cyanobacteria to perform oxygenic photosynthesis is thought to have converted the early reducing atmosphere into an oxidizing one, which dramatically changed the composition of life-forms on earth by stimulating biodiversity and leading to the near extinction of oxygen intolerant organisms. Their prokaryotic type nucleus definitely sets them apart from the eukaryotic algae. Although some bacteria are phototropic, the difference between phototropic bacteria and cyanobacteria is that the cyanobacteria have chlorophyll *a* and the phototropic bacteria do not have. Bacteriochlorophyll is the photosynthetic pigment in the phototropic bacteria. Over thousand species have been reported (Fig. 1.15). Aquatic cyanobacteria are probably best known for the extensive and highly visible blooms that can form in both freshwater and the marine environment and can have the appearance of blue-green paint or scum. The association of toxicity with such blooms has frequently led to the closure of recreational waters when blooms are observed.

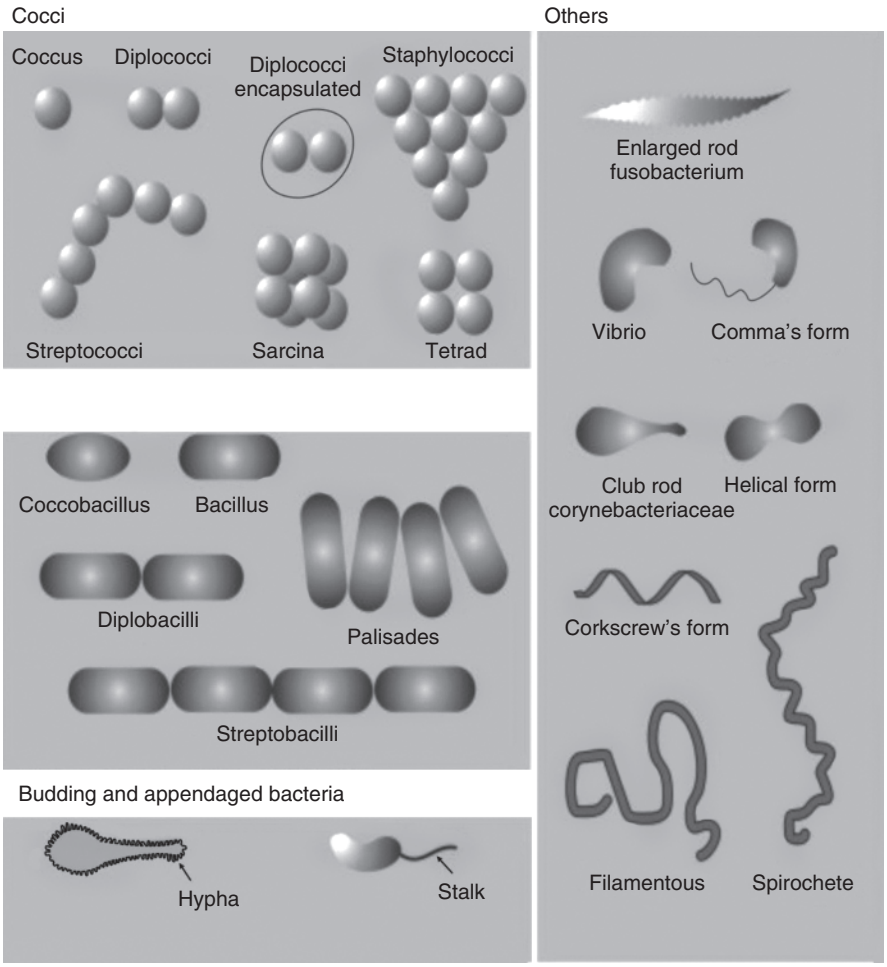

Figure 1.14. Bacterial morphology.

Cyanobacteria account for 20–30% of the earth's photosynthetic productivity and convert solar energy into biomass stored chemical energy at the rate of ~450 TW. Cyanobacteria utilize the energy of sunlight to drive photosynthesis, a process where the energy of light is used to split water molecules into oxygen, protons, and electrons. While most of the high energy electrons derived from water are utilized by the cyanobacterial cells for their own needs, a fraction of these electrons are donated to the external environment via electrogenic activity. Cyanobacterial electrogenic activity is an important microbiological conduit of solar energy into the biosphere.

Cyanobacteria are the only group of organism (Fig. 1.15) that are able to reduce nitrogen and carbon in aerobic conditions, a fact that may be responsible for their evolutionary and ecological success. The water-oxidizing photosynthesis is accomplished by coupling the activity of photosystem (PS) II and I (Z-scheme). In anaerobic conditions, they are also able to use only PS I—cyclic photophosphorylation with electron donors other than water (hydrogen sulfide, thiosulphate, or even molecular hydrogen)

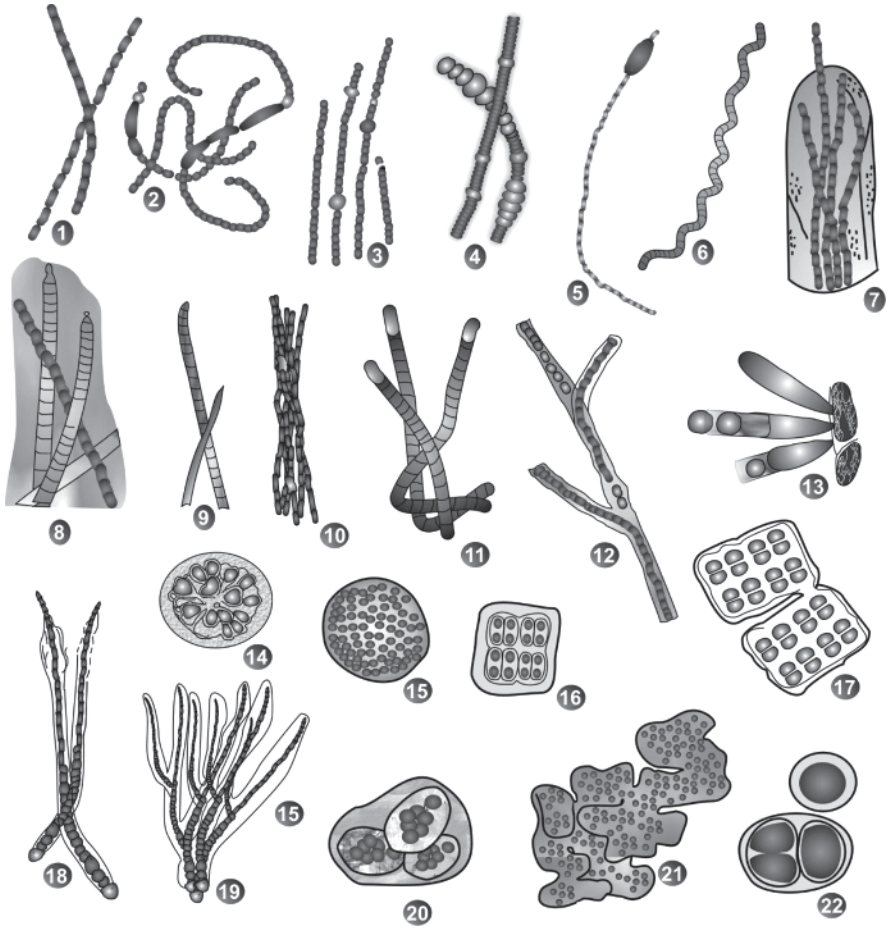

Figure 1.15. Schematic diagram of different forms of cyanobacteria (blue-green algae): (1) *Anabaena* (350×), (2) *Anabaena* (350×), (3) *Anabaena* (175×), (4) *Nodularia* (350×), (5) *Cylindrospermum* (175×), (6) *Arthrospira* (700×), (7) *Microcoleus* (350×), (8) *Phormidium* (350×), (9) *Oscillatoria* (175×), (10) *Aphanizomenon* (175×), (11) *Lyngbya* (700×), (12) *Tolpothrix* (350×), (13) *Entophysalis* (1000×), (14) *Gomphosphaeria* (1000×), (15) *Gomphosphaeria* (350×), (16) *Agmenellum* (700×), (17) *Agmenellum* (175×), (18) *Calothrix* (350×), (19) *Rivularia* (175×), (20) *Anacystis* (700×), (21) *Anacystis* (175×), (22) *Anacystis* (700×).

just like purple photosynthetic bacteria. Furthermore, they share an archaeal property, the ability to reduce elemental sulfur by anaerobic respiration in the dark. Their photosynthetic electron transport shares the same compartment as the components of respiratory electron transport. Their plasma membrane contains only components of the respiratory chain, while the thylakoid membrane hosts both respiratory and photosynthetic electron transport. Cyanobacteria have an elaborate and highly organized system of internal membranes that function in photosynthesis. They are present in almost all moist environments from the tropics to the poles, including both freshwater and marine. The different colors are due to the presence of the pigments like chlorophyll *a*, carotene, xanthophylls, *c*-phycocyanine that are produced in varying proportions. Phycocyanin and red

c-phycoerythrin are unique to cyanobacteria and red algae. Cellular structure is considerably different from that of the eukaryotic algae. Nuclear membranes are absent in cyanobacteria. The nuclear material consists of DNA granules in a more or less colorless area in the center of the cell. Unlike the algae, the pigments of the cyanobacteria are not contained in the chloroplast; instead they are located in granules . Phycobilisomes are attached to membranes that permeate the cytoplasm. According to endosymbiotic theory, chloroplasts in plants and eukaryotic algae have evolved from cyanobacteria via endosymbiosis. Owing to their ability to fix nitrogen in aerobic conditions they are often found as symbionts with a number of other groups of organisms such as fungi (lichens), corals, pteridophytes (*Azolla*), and angiosperms (*Gunnera*), and provide energy to the host.

1.3.3.2. Archaea

Archaea are microbes: most live in extreme environments (called *extremophiles*; Delong, 1992) and others live in normal temperatures and salinities, some even live in our guts. Some extremophile species love heat; they like to live in boiling water, like the geysers of Yellowstone Park, and inside volcanoes (Wächtershäuser, 2006). They like the heat so much that it has earned the nickname "thermophile," which means "loving heat," and it would probably freeze to death at room temperature. Other extremophile Archaea love to live in very salty, called *hypersaline*, environments. They are able to survive in these extreme places where other organisms cannot. These salt loving archaea are called *halophiles*.

Archaea was originally thought to be just like bacteria, but archaea is a much different and simpler form of life. It may also be the oldest form of life on earth (Wächtershäuser, 2006). Archaea requires neither sunlight for photosynthesis as do plants, nor oxygen. Archaea absorbs CO_2, N_2, or H_2S and gives off methane gas as a waste product the same way humans breathe in oxygen and breathe out carbon dioxide. The relationship between archaea and eukaryotes remains problematic.

Complicating factors include claims that the relationship between eukaryotes and the archaeal phylum Euryarchaeota is closer compared to the relationship between the Euryarchaeota and the phylum Crenarchaeota and the presence of archaean-like genes in certain bacteria, such as *Thermotoga maritima*, from horizontal gene transfer. The leading hypothesis is that the ancestor of the eukaryotes diverged early from the archaea, and that eukaryotes arose through fusion of an archaeon and eubacterium, which became the nucleus and cytoplasm; this accounts for various genetic similarities but runs into difficulties explaining cell structure.

1.3.4. Eucarya

All members of the living world except the prokaryotes are in the domain Eukarya, and all members consist of eukaryotic cells. The microbial world is composed of single-celled members of the Eucarya as well as their close multicellular relatives. These members include algae, fungi, and protozoa. Algae and protozoa are also referred to as *protists*. The Eukarya are the structurally simple eukaryotes that lack mitochondria and some other key organelles. Most of these eukaryotes are metabolically deficient and are pathogenic parasites of humans and other animals. Like prokaryotes, there exists a diverse array of eukaryotic microorganisms.

Some of these such as algae are phototropic; they contain chlorophyll-rich organelles called *chloroplast* and live in an environment that contains only a few minerals, carbohydrates, and CO_2 and light.

The Eucarya are usually classified into four kingdoms: plants, animals, fungi, and protists. Kingdom under domain Archaea is Archaeabacteria and kingdom under domain Bacteria is Eubacteria. Eukarya is the one which has four kingdoms under, namely Plantae, Animalia, Protista, and Fungi. The first three of these correspond to phylogenetically coherent groups as well. However, the eukaryotic protists do not form a group, but rather comprise many phylogenetically disparate groups (including slime molds, multiple groups of algae, and many distinct groups of protozoa). Just as molecular analyses were required to see the natural relationships among prokaryotes, they are also allowing us to infer relationships among these nonplant, nonanimal, nonfungus eukaryotes (Figs. 1.16 and 1.17).

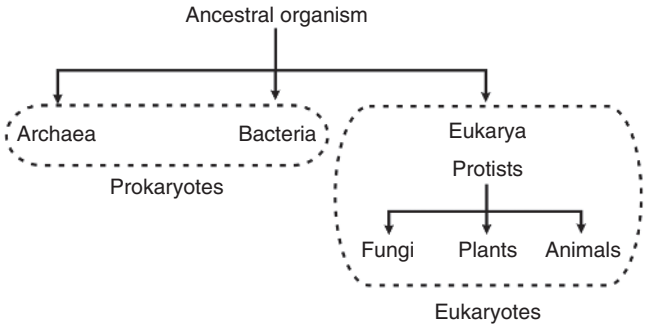

Figure 1.16. Interrelation between prokaryotes and eukaryotes.

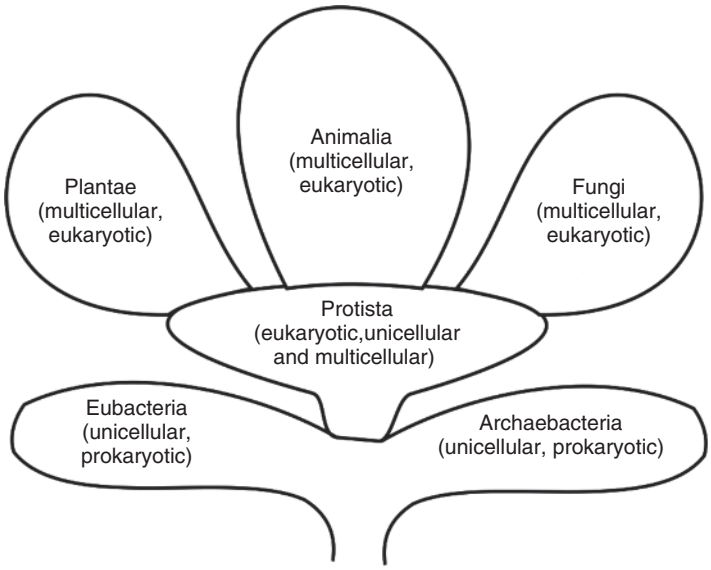

Figure 1.17. Phylogenetic origin of Eukarya.

Many groups of fungi are important. In addition to providing yeast for making bread and fermenting sugar to alcohol, the fungi include the causative agents responsible for conditions such as blight, ergot, rot, rust, smut, wilt, ringworm, and athlete's foot. Although it might seem that it is straightforward to identify an organism as a fungus, this is not always the case. The unambiguous identification of the opportunistic pathogen, *Pneumocystis* (which has not been grown in cultures), as a fungus required analysis of its ribosomal RNA. The "eukaryotic protists" also provide many organisms of clinical importance, including *Crithidia, Trypanosoma, Plasmodium, Amoeba*, and *Giardia*.

Eukarya differ fundamentally from the Bacteria and Archaea in terms of the organization of the genetic machinery with multiple chromosomes being housed within a nucleus bounded by a double membrane. This unique and complex organization must be explained by any theory on the origin of the Eukarya. The theory presented here delivers an explanation in addition to solving the lipid problem. It explains the eukaryal genetic machinery as an automatic, irreversible, almost deterministic consequence of the endosymbiosis, whereby a precell was converted into the nucleus. The following 11 features that are shared by all extant Eukarya may be explained by the historic origin of the nucleus in an endosymbiotic precell within a bacterial host:

1. The nonorganellar genome is polychromosomal and the chromosomes are housed inside the nucleus. This can be explained by the hypothesis that a polychromosomal precell turned into the nucleus, whereby the precell chromosomes became trapped and separated from the outer cell membrane by the nuclear double membrane. Therefore, their replication could not become linked with the outer cell membrane during cell division. This means that there was no selective advantage in their unification into one large circular chromosome as was the case with the Bacteria and Archaea.
2. The nuclear membrane is endowed with pores and connected with an endoplasmic reticulum (Moreira and Lopez-Garcia, 1998).
3. The nonorganellar chromosomes are linear as opposed to the circular bacterial and archaeal chromosomes. This can be explained by their possible origin in the linear intermediates of the hypothesized rolling circle replication of the precell chromosomes, perhaps with a multiplication in length.
4. Reproduction is based on a process of mitosis. The higher Eukarya, such as plants and animals, have an open mitosis, while all others have a closed mitosis. This finds a simple explanation in the hypothesis that the nucleus originated from a polychromosomal precell, which means that closed mitosis is original and open mitosis is a derived process.
5. The nuclear chromosomes contain genes of bacterial origin. Approximately 75% of these bacterial genes in the nuclear chromosomes are clearly of nonorganellar vintage (Esser et al., 2004). This can be explained with an evolutionary process, whereby the outer bacterial chromosomes were lost and their indispensable genes became relocated into the nuclear chromosomes.
6. The intermediary and energy metabolism is restricted to the outer cytoplasm. As a corollary, protein synthesis by translation is also restricted to the outer cytoplasm, that is, to exist in close quarters with the metabolism, where the enzymes are needed. This can be explained by the hypothesis that in the endosymbiosis, it would have been the bacterial host cell, which took charge of intermediary and

energy metabolism, with the advantage that this metabolism moved closest to the influx of nutrients. Incidentally, some translation appears to have remained to this day inside the nucleus (Brogna, 2001). This is simply explainable as a leftover from precell days.

7. Transcription occurs exclusively inside the nucleus and the mRNAs become short and capped. They are exported through the nuclear pores into the cytoplasm. This can be explained by the hypothesis that the expression of the genes became separated from their original location in the precell turned nucleus and that the requirement of their transport through the nuclear pores did not permit preservation of long operons as they are active in extant Bacteria and Archaea.
8. The nonorganellar ribosomes are synthesized in a peculiar manner. The rRNAs are synthesized inside the nucleus. The ribosomal proteins are synthesized outside the nucleus. The ribosomal proteins are synthesized outside the nucleus in the cytoplasm. Some of the ribosomal proteins are then imported into the nucleus for assembly with the ribosomal RNAs to form incomplete ribosomal subunits. The incomplete ribosomal subunits are then exported through the pores of the nuclear membrane into the cytoplasm, where they are completed by the addition of further ribosomal proteins. The completed ribosomes are then used in the cytoplasm for translation. This roundabout pattern can be explained by the hypothesis that the incipient precell endosymbiont inside the bacterial host would have maintained a precellular process of translation for some time. The incomplete ribosomal subunits would simply reflect their origin in primitive precellular ribosomes, thus give us insights into the most ancient ribosomes and their early evolution.
9. Most genes in the nuclear chromosomes are infested with introns of unknown origin. This infestation with introns is not lethal or even particularly deleterious due to a peculiar processing (maturation) of the primary mRNAs inside the nucleus. The introns are spliced out and the remaining mRNA segments are interconnected to form a mature, functional mRNA. This can be explained by the hypothesis that a physical isolation of the intranuclear, precell-derived space from the extranuclear bacteria-derived cytoplasmic space would have ensued with endosymbiosis. This means that the intranuclear space would have become a safe compartment for intron splicing so that immature intron-laden mRNAs would have been prevented from entering the cytoplasm, thereby the metabolism would have been protected from being messed up with products of a translation of immature mRNAs.
10. The genes for the intermediary metabolism are most closely related to the bacteria while the genetic machinery is most closely related to and actually more complex than the genetic machinery of the archaea. This can now be explained with reference to the hypothetical phylogeny. The Bacteria are shown as diverging first from relatively primitive precells. The Archaea are shown as diverging later from evolutionarily more advanced precells. The Eukarya are shown as diverging last (by endosymbiosis) from precells that would have been still farther evolved than the precells, from which the Archaea originated. This rendered the precell-derived genetic machinery of the Eukarya similar to, but more evolved than, the genetic machinery of the Archaea. The close relationship of the metabolic genes of the Eukarya with those of the Bacteria is simply due to their bacterial origin.

11. The genetic machinery inside the nucleus has somewhat "primitive" features with numerous small RNAs being involved in replication, transcription, and mRNA maturation. This can now be explained by the hypothesis that these "primitive" features would date back as leftovers to the workings of the "primitive" precells that were involved in endosymbiosis.

With these 11 explanatory points, the presentation of theory of the origin of the Eukarya by endosymbiosis of precells and wall-less bacteria is completed.

1.3.5. Algae

The subkingdom Algae includes all the photosynthetic eukaryotic organisms in the protista. Being true protista, they lack tissue differentiation. In Whittaker's five-kingdom system, some algae have been included with protozoan. The algae may be unicellular, colonial, or filamentous. The undifferentiated algal structure is often referred to as *thallus*. They lack the stem, root, leaf structure that result from tissue specialization. Algae are organisms that use light energy to convert CO_2 and H_2O to carbohydrates and other cellular products with the release of oxygen. Algae contain chlorophyll *a* which is necessary for photosynthesis. In addition, many other algae contain other pigments that extend the range of light waves that can be used by these organisms for photosynthesis. Algae include both microscopic and macroscopic multicellular organisms (Figs. 1.18–1.20).

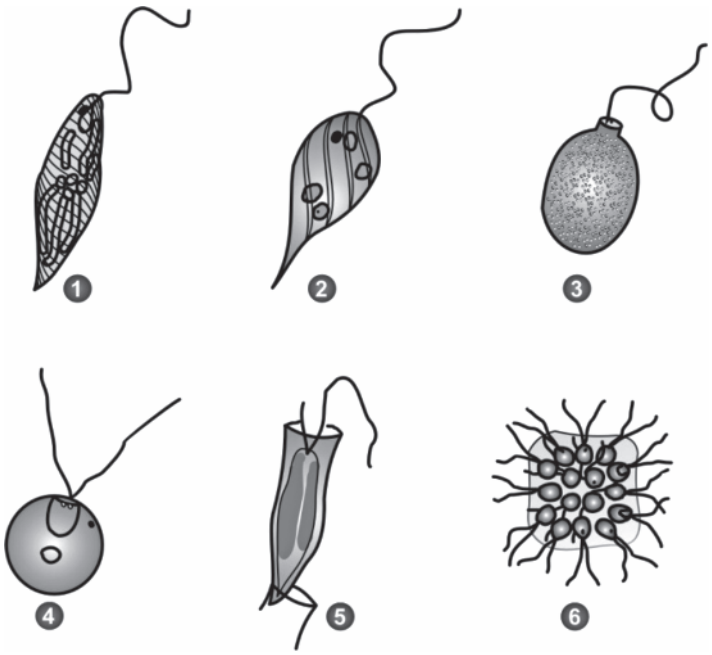

Figure 1.18. Microscopic view of flagellated algae: (1) *Euglena* (700×), (2) *Phacus* (1000×), (3) *Trachelomonas* (1000×), (4) *Chlamydomonas* (1000×), (5) *Dinobyron* (1000×), (6) *Gonium* (350×).

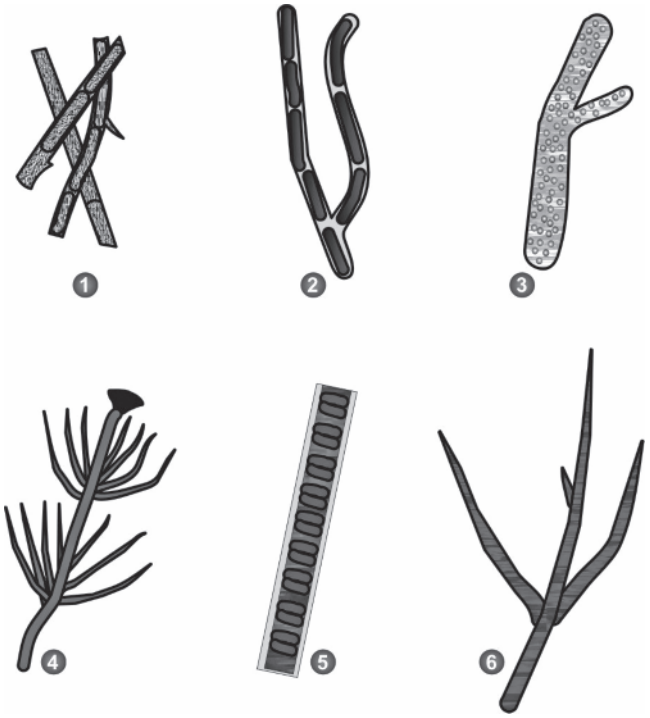

Figure 1.19. Microscopic view of filamentous algae: (1) *Rhizoclonium* (175×), (2) *Cladophora* (100×), (3) *Vaucheria* (100×), (4) *Chara* (3×), (5) *Zygnema* (175×), (6) *Stigeoclonium* (100×).

Algae, however, lack a well-organized vascular system. Algae do not directly infect humans but some produce toxins that cause paralytic shell fish poisoning. Some of these toxins do not cause illness in the shellfish that feed on the algae but accumulate in their tissues and when eaten by man cause nerve damage. As one of the primary producers of carbohydrate and other cellular products, the algae are essential in the food chains of the world. In addition, they produce a large proportion of the oxygen in the atmosphere.

These microorganisms are universally present where ample moisture, favorable temperature, and sufficient sunlight exist. Although a great majority of them live submerged in water, some grow on soil, others grow on the bark of trees or on the surface of the rocks. Algae have distinct, visible nuclei and chloroplast. Chloroplast is the organelle that contains chlorophyll *a* and other pigments. Photosynthesis takes place within these bodies. The size, shape, distribution, and number of chloroplasts vary considerably from species to species. In some instances a single chloroplast may occupy most of the cell space.

Although there are seven divisions of algae, only five will be listed here. Since the two groups cryptomonads and red algae are usually not encountered in freshwater ponds.

1.3.5.1. Euglenophycophyta (Euglenoids)

All members of this division are flagellated and appear to be intermediates between the algae and protozoa. Protozoan-like characteristics seen in euglenoids are the absence of

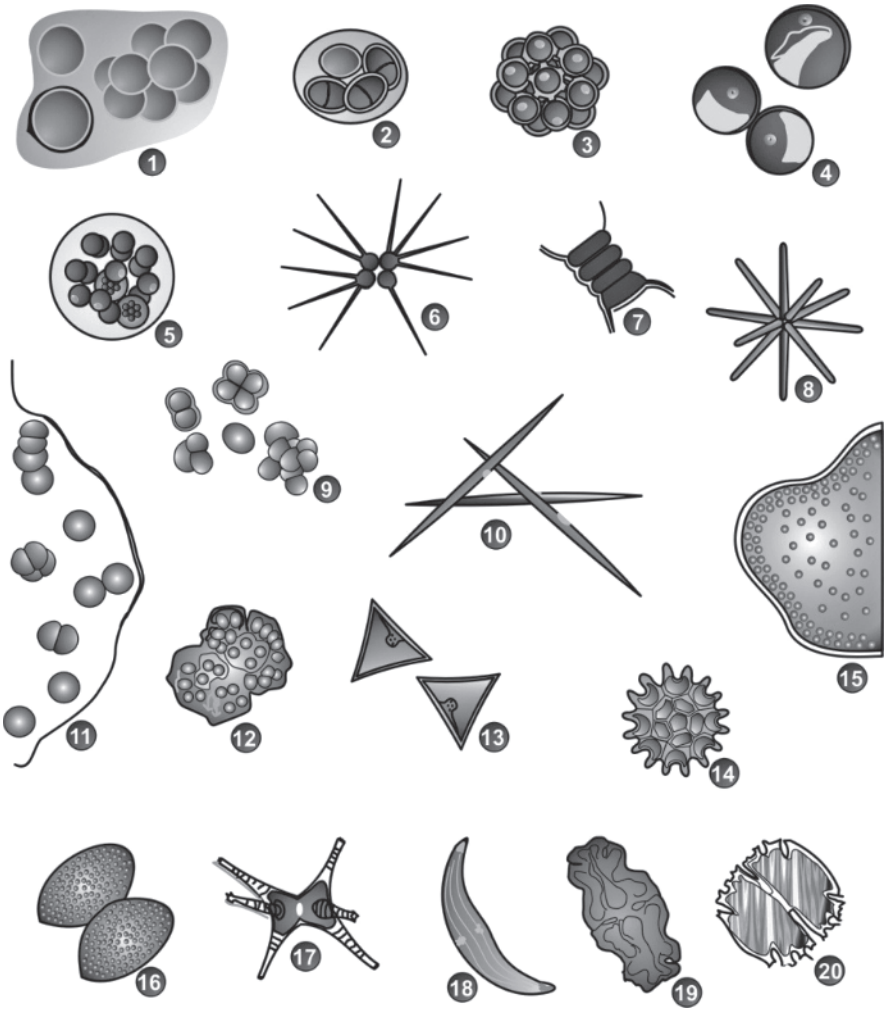

Figure 1.20. Microscopic view of nonfilamentous and nonflagellated algae: (1) *Chlorococcum* (700×), (2) *Oocystis* (700×), (3) *Coelastrum* (350×), (4) *Chlorellia* (350×), (5) *Sphaerocystis* (350×), (6) *Micractinium* (700×), (7) *Scendesmus* (700×), (8) *Actinastrum* (700×), (9) *Phytoconis* (700×), (10) *Ankistrodesmus* (700×), (11) *Pamella* (700×), (12) *Botryococcus* (700×), (13) *Tetraedron* (1000×), (14) *Petraspora* (100×), (15) *Tetraspora* (100×), (16) *Staurastrum* (700×), (17) *Staurastrum* (350×), (18) *Closterium* (175×), (19) *Euastrum* (350×), (20) *Micrasterias* (175×).

cell wall, presence of gullet, ability to assimilate organic substances, and absence of chloroplast in some species. The absence of cell wall makes these protists very flexible. Instead of cell wall, they possess a semirigid outer pellicle, which gives the organism a definite form. Photosynthesis types contain chlorophyll *a* and *b*, and they always have a red stigma or eyespot that is light sensitive. Their characteristic food storage is a lipopolysaccharide, paramylum. The photosynthetic euglenoids can be bleached experimentally by various means in the laboratory. The colorless forms that develop, however, cannot be induced to revert back to phototrophy.

1.3.5.2. Chlorophycophyta (Green Algae)

The green algae are present in ponds. They are grass green in color, resembling the euglenoids in having chlorophyll *a* and *b*. They differ from the euglenoids in that they synthesize starch instead of paramylum for food storage.

The diversity of this group is too great to explore; however, the small flagellated *Chlamydomonas* appears to be the archetype of the entire group. Many colonial forms such as *Pandorina, Eudorina, Gonium, Volvox* consist of organisms similar to *Chlamydomonas*. It is the consensus that all the filamentous algae that have evolved from this flagellated form are Chlorophycophyta. A unique group of green algae are the desmids. With the exceptions of a few species, the cells of desmids consist of two similar halves or semicells. The two halves usually are separated by a constriction, the isthmus.

1.3.5.3. Chrysophycophyta (Golden Brown Algae)

This large diversified division consists of over 6000 species (Fig. 1.21). They differ from the euglenoids and green algae in food storage in the form of oils and leucosin, and in pigments such as chlorophyll *a* and *c* and fucoxanthin, a brownish pigment. It is a combination of fucoxanthin, other yellow pigments, and chlorophylls that causes most of these algae to appear golden brown. Representatives of this division are *Chrysococcus, Synura*, and *Dinobryon*, which are typical flagellated chrysophycophytes. *Vaucheria* and *Tribonema* are the only filamentous chrysophycophytes. All these organisms fall under a special category of algae called *diatoms*. The diatoms are unique in that they have hard cell walls of pectin, cellulose, or silicon oxide that are constructed in two halves. The two halves fit like a lid and box. Skeletons of dead diatoms accumulate on the ocean

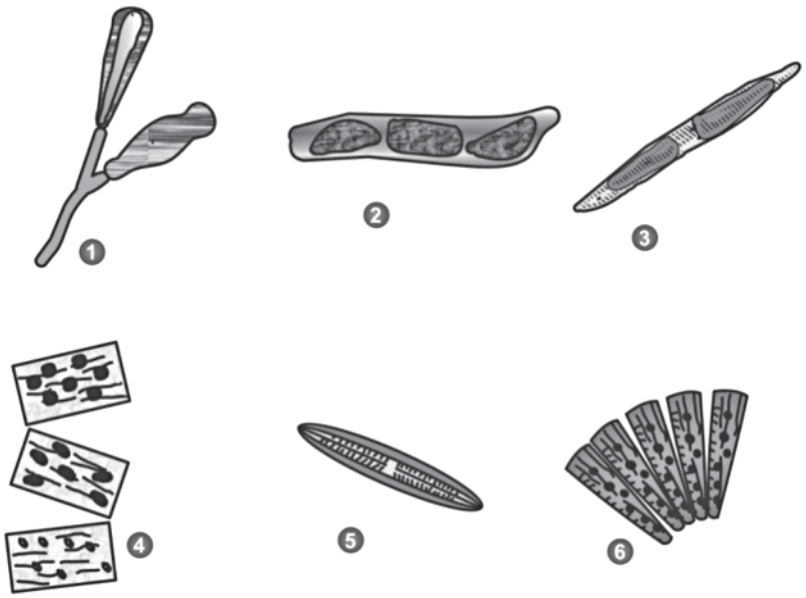

Figure 1.21. Microscopic view of diatoms: (1) *Gomphonema* (175×), (2) *Cymbella* (175×), (3) *Nitschia* (1500×), (4) *Tabellaria* (1000×), (5) *Navicula* (750×), (6) *Meridion* (750×).

bottom to form diatomite or diatomaceous earth that is commercially available as an excellent polishing compound. It is postulated that much of our petroleum compounds may have been formulated by the accumulation of oil from dead diatoms over millions of years.

1.3.5.4. Lichens (Symbionts)

Lichens are composite organisms consisting of a symbiotic association of a fungus (the mycobiont) with a photosynthetic partner (the photobiont or phycobiont), usually either a green alga (commonly *Trebouxia*) or a cyanobacterium (commonly *Nostoc*). The morphology, physiology, and biochemistry of lichens are very different from those of the isolated fungus and cyanobacteria in cultures (Fig. 1.22). Lichens occur in some of the most extreme environments on earth: arctic tundra, hot deserts, rocky coasts, and toxic slag heaps. However, they are also seen abundant as epiphytes on leaves and branches in rain forests and temperate woodland, on bare rock, including walls and gravestones, and on exposed soil surfaces (e.g., *Collema*) in otherwise mesic habitats. Lichens are widespread and maybe long-lived; however, many are also vulnerable to environmental disturbance, and may be useful to scientists in assessing the effects of air pollution, ozone depletion, and metal contamination. Lichens have also been used in making dyes and perfumes, as well as in traditional medicines. Thallus is the body of the lichen. The fungal hyphae (filaments) branch and then fuse together (anastomose) to form a mesh of hair-like threads. The top surface is normally a layer of tightly packed hyphae known as *cortex* covering below the algal layer where the photobiont lives. Below this is the medulla an area of loose hyphae in which nutrients are stored. Sometimes, a lower cortex exists; in others, the medulla rests on the surface. Filamentous lichens consist of chains of algal cells wrapped around with fungal hyphae. Cladonia are a successful group of lichens, which have a primary thallus and a secondary thallus. The primary thallus is small and clings closely to the substrate while the secondary thallus is a shrubby growth, for example, *fruticose lichens*. Once the lichen is established, the primary thallus

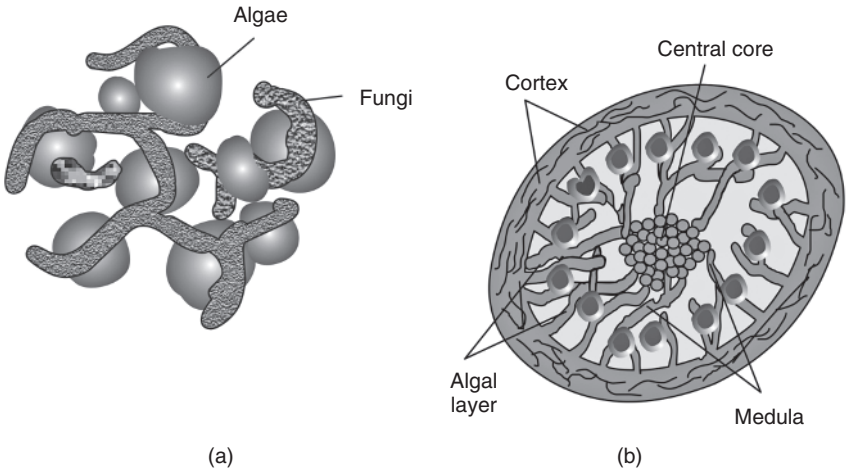

Figure 1.22. (a) Symbiotic association in lichen, (b) TS of fruticose lichens.

often dies off. Lichens have been classified on the basis of their morphology, symbiotic relationship, and the type of structure:

1. *Crustose Lichens*. These are classified on the basis of forming a crust on the surface of the substrate on which they are growing. This crust can be quite thick and granular or actually embedded within the substrate. In this latter case, the fruiting bodies still rise above the surface. In many crustose lichens the surface of the thallus breaks up into a cellular, crazy-paving-like pattern. Crustose lichens tend to grow out from their edges and have their fruiting bodies in their center. Crustose lichens are very difficult to remove from their substrates.
2. *Squamulose Lichens*. These have a portion of their thallus lifted off the substrate to form "squamules." They are otherwise similar to crustose lichens in that they possess an upper cortex but no lower cortex (Fig. 1.23).
3. *Foliose Lichens*. These have an upper and lower cortex. They are generally raised to some extent above the substrate but connected to it by rhizines (specialized root-like hyphae). They are easier to remove from their substrate.
4. *Fruticose Lichens*. These are attached to their substrate by a single point and rise, or, more usually, dangle from this and are also known as shrubby lichens. Some foliose lichens can be stubby like fruticose lichens; however, close examination will reveal that the algal part exists only on one side of the flattish thallus, whereas in fruticose lichens it exists as a ring around the thallus, even when it is flattened as in *Ramalina* sp.
5. *Leprose Lichens*. These are an odd group of lichens that have never been observed to produce fruiting bodies and have not yet been identified properly or, at least, not yet given full scientific names. They not only lack an inner cortex but also lack an outer one, that is, no cortex, only an algal cell layer and sometimes a weakly defined medulla (Fig. 1.24).

1.3.6. Fungi

Fungi comprise of a large group of eukaryotic nonphotosynthetic organisms that include such diverse forms as slime molds, water molds, mushrooms, puff balls, bracket fungi, yeasts, and molds. They belong to the kingdom Mycetae and the study is called *mycology*. Myceteae consists of three divisions: Gymnomycota, the slime moulds; mastigomycota,

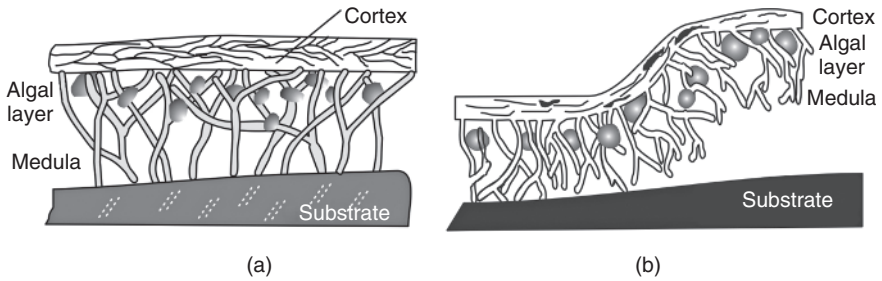

Figure 1.23. (a) Crustose lichen and (b) squamulose lichen.

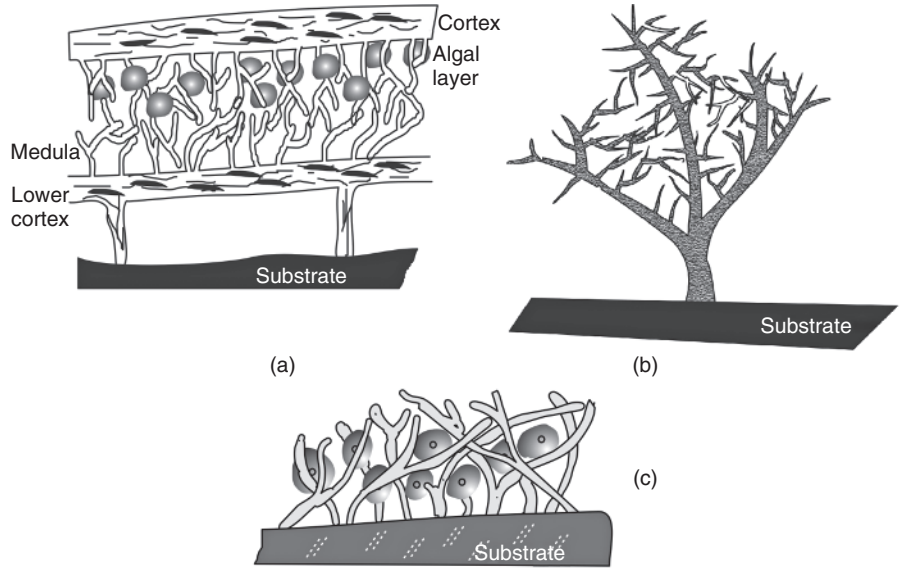

Figure 1.24. (a) Foliose lichen, (b) fructicose lichen, (c) leprose lichen.

Figure 1.25. Schematic diagram of fungi: (a) *Rhizopus*, (b) *Penicillium*, (c) *Polyporus*, (d) *Cercospora*.

the water moulds; and amastigomycota, the yeast, the molds, bracket fungi, and so on. Most fungi are aerobic or facultative anaerobic. Only a few fungi are anaerobic. A large number of fungi cause diseases in plants. Fortunately, only a few species cause diseases in animals and humans. Along with bacteria, fungi are the principal decomposers of carbon compounds on the earth. The decomposition releases carbon dioxide into the atmosphere and nitrogen compounds into the soil, which are then taken up by plants and converted into organic compounds. Without this breakdown of organic material, the world would quickly be overrun with organic waste.

Fungi may be saprophytic or parasitic and unicellular or filamentous organism (Fig. 1.25). Some organisms such as slime molds are borderline between fungi and protozoa in that amoeboid characteristics are present and fungi-like spores are produced. The distinguishing characters of the group as a whole are that they are eukaryotic, nonphotosynthetic, lack tissue differentiation, have cell walls made up of chitin and other polysaccharides, and are propagated by spores both sexually and asexually.

Species within the Amastigomycota may have cottony or mold-like appearance, or moist or yeasty appearance that set them apart. Some species exist as molds under some conditions and some as yeast-like under other conditions. Such species are said to be dimorphic or diphasic.

1.3.6.1. Molds

The molds have microscopic filaments called *hyphae*. If the filaments have cross walls, it is referred to as *septate hyphae*. If no cross walls are present, it is called *nonseptate* or *aseptate*. Actually, most of the fungi that are classified as being septate are incompletely septate, have central openings that allow the streaming of the cytoplasm from one compartment to another. A mass of unmeshed microscopic hyphae is called *mycelium*. Two kinds of asexual spores are seen in molds: sporangia and conidia. Sporangiospores are spores that are formed within a sac called *sporangium*. The sporangia are attached to stalks called *sporangiospore*. Conidia are asexual spores that form on specialized hyphae called *conidiospore*. If the conidia are small they are called *microconidia*; large multicellular conidia are known as *macroconidia*. The four types of conidia are as follows:

Phialospores. Conidia of this type are produced by vase shaped cells called phialids. *Penicillium* and *Gliocadium* are produced by this type.

Blastoconidia. Conidia of this type are produced by budding from the cells of the preexisting conidia as in *Cladosporium*, which typically has lemon-shaped spores.

Arthospores. This type of conidia forms by separation from the preexisting hyphal cells, for example, *Oospora*.

Chlamydomonas. These spores are large, thick-walled, round or irregular structure formed within or at the ends of hyphae. Common to most fungi, they generally form on old cultures, for example, *Candida albicans*.

Sexual spores: Three kinds of sexual spores are seen in molds: zygospores, ascospores, and basidospores. Zygospores are formed by the union of nuclear material from the hyphae of the two different strains. Ascospores, on the other hand, are sexual spores produced in enclosures, which may be oval sacs or elongated tubes. Basidiospores are sexually produced on club-shaped bodies called *basidia*. A basidium is considered by some to be a modified type of ascus.

1.3.6.2. Yeast

Unlike molds, yeast does not have true hyphae. Instead, they form multicellular structures called *pseudohyphae*. The only asexual spores formed are called *blastospores* or *bud*. These spores form as an out pouching of the cell by the budding process, resulting in the formation of *pseudohyphae*.

Division Amastigomycota consists of four subdivisions: Zygomycotina, Ascomycotina, Basidiomycotina, and Deuteromycotina.

ZYGOMYCOTINA. These fungi have septate hyphae and produce zygospores. They also produce sporangiospores. *Rhizopus, Mucor*, and *Syncephalastrum* are representative genera of this subdivision.

ASCOMYCOTINA. Since all fungi in this subdivision produce ascospores, they are grouped into one class. They are commonly referred to as the *Ascomycetes* and are also called *sac fungi*. All of them have septate hyphae and most of them have chitinous walls (Fig. 1.26).

Fungi in this group produce a single ascus, *ascomycete yeasts*. Other ascomycetes produce numerous asci in complex flask-shaped fruiting bodies (*perithecia* or *pseudothecia*), in cup-shaped structures, or in hollow spherical bodies, as in powdery mildews, *Eupenicillium* or *Talaromyces*, the sexual stages for *Penicillum*.

BASIDIOMYCOTINA. All fungi in this subdivision belong to one class, the Basidiomycetes. Puffballs, mushrooms, smuts, rust, and shelf fungi on tree branches are also basidiomycetes. The sexual spores of this class are basidiospores.

DEUTEROMYCOTINA. This fourth division of the Amastigomycotina is an artificial group that was created to place any fungi that has not been shown to have some means of sexual reproduction. Often, species that are relegated to this division remain here for only a short period of time; as soon as the right condition has been provided for the sexual spores to form, they are reclassified into one of the subdivisions. Sometimes, however, the sexual and asexual stages of the fungus are discovered and named separately by different mycologists, with the result that a single species acquires two different names. Generally, there is a switch over to the sexual stage name. Members of this group are called *fungi imperfecti* or *deuteromycetes*.

1.3.7. Protozoa

The phylum Protozoa consists of about 15,000 species of protozoans. The phylum consists of microscopic organisms in which all the vital activities are performed by a single cell, commonly referred to as *single-celled organisms*. They are cosmopolitan in distribution and include aquatic (freshwater and marine) forms too. Phylum Protozoa includes free-living animals (Fig. 1.27) such as *Euglena, Paramecium, Amoeba, Noctiluca*, and *Elphidium* and parasitic animals such as *Plasmodium* species, *Monocystic, Entamoeba, Trypanosoma*, and *Giardia*. The organisms of this phylum greatly vary in shape, size, locomotory organelles, and method of reproduction.

The subkingdom Protozoa includes single-celled organisms; however, some of them do form colonial aggregates. Externally, the cells are covered with cell membranes or a pellicle, cell walls are absent, and distinct nuclei with nuclear membranes are present. Specialized organelles such as contractile vacuoles, cytostomes, mitochondria, ribosomes, flagella, and cilia may also be present.

All protozoa produce cysts, which are resistant dormant stages that enable them to survive drought, heat, and freezing. They produce asexually by cell division and exhibit various degrees of sexual reproduction. The subkingdom Protozoa is divided into three phyla: Sarcomastigophora, Ciliophora, and Apicomplexa. Type of locomotion plays an important role in classifying them. A brief description of each phylum follows.

1.3.7.1. Sarcomastigophora

Members of this phylum are divided into two subphyla: Sarcodina and Mastigophora. Members of the subphylum Sarcodina (*Amoeba*), move about by the formation of flowing

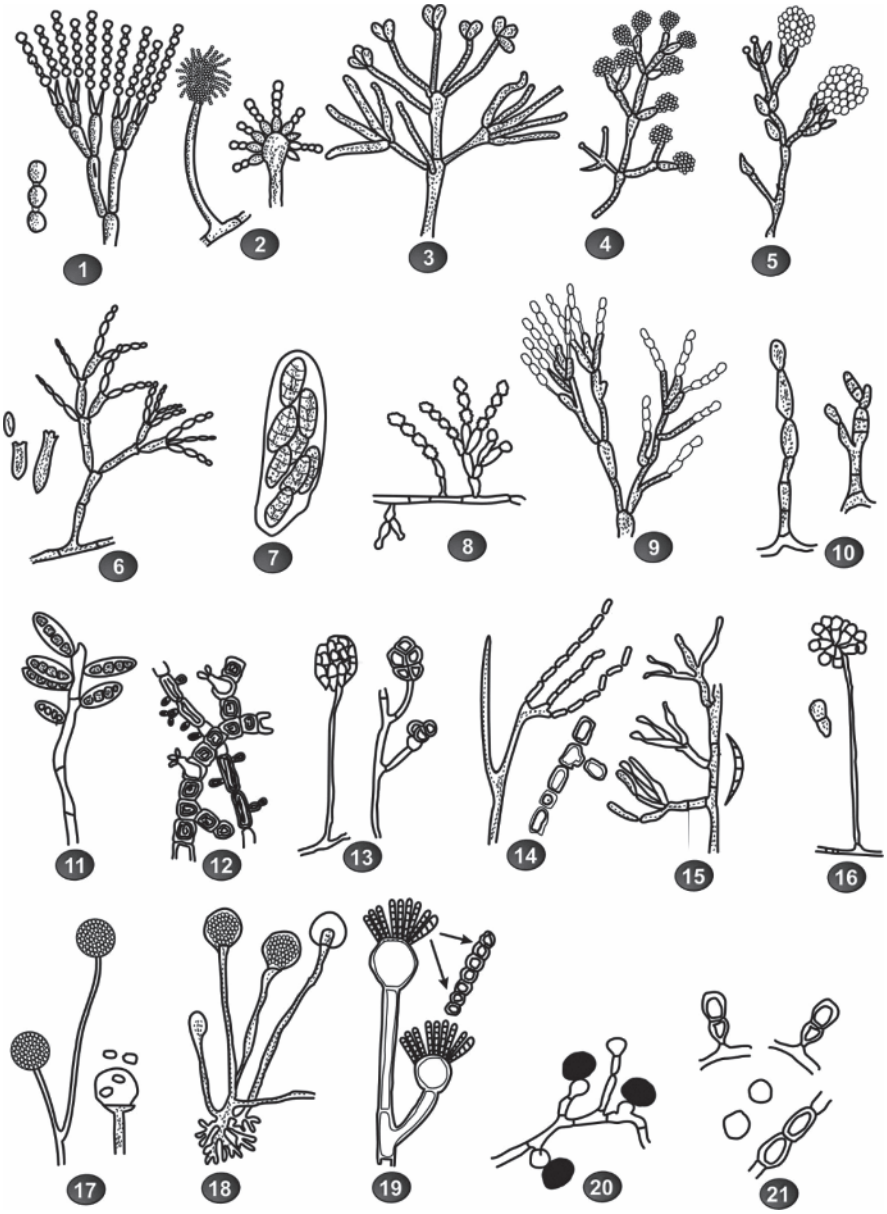

Figure 1.26. Microscopic appearance of some more common molds: (1) *Penicillium*, (2) *Aspergillus*, (3) *Verticillium*, (4) *Trichoderma*, (5) *Gliocadium*, (6) *Cladosporium*, (7) *Pleospora*, (8) *Scopulariopsis*, (9) *Paecilomyces*, (10) *Alternaria*, (11) *Bipolaris*, (12) *Pullularia*, (13) *Diplosporium*, (14) *Oospora*, (15) *Fusarium*, (16) *Trichothecium*, (17) *Mucor*, (18) *Rhizopus*, (19) *Syncephalastrum*, (20) *Nigrospora*, (21) *Montospora*.

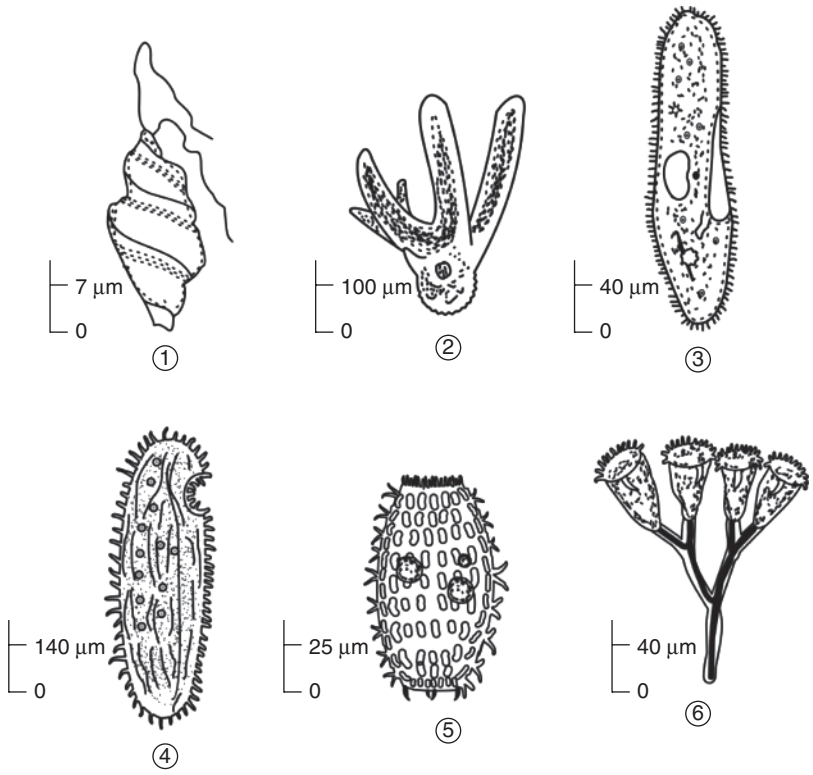

Figure 1.27. Microscopic view of different protozoans: (1) *Heteronema*, (2) *Amoeba*, (3) *Paramecium*, (4) *Loxodes*, (5) *Coleps*, (6) *Zoothamnium*.

protoplasmic projections called *pseudopodia*. The formation of pseudopodia is commonly referred to as *amoeboid movement*. The subphylum Mastigophora (*Zooflagellates*) possess whip-like structures called *flagella*. There is a considerable diversity among members of this group.

1.3.7.2. Ciliophora

Microorganisms of this phylum are undoubtedly the most advanced and structurally complex of all protozoans. Evidence seems to indicate that they have evolved from the zooflagellates. Movement and procuring food are accomplished with a short hair-like structure called *cilia*.

1.3.7.3. Apicomplexa

This phylum has only one class, the Sporozoa. Members of this phylum lack locomotor organelles, and are all internal parasites. Their life cycle includes spore forming stages. *Plasmodium*, the malaria parasite, is a significant pathogenic sporozoan of humans.

1.3.7.4. Genome of Protozoan

The completion of five new protozoan parasite genomes led to an improved understanding of their biology, and comparative genomics shed light on the diversity of protozoan genome and evolution. *Entamoeba histolytica* is a human gut parasite that causes amebiasis, a significant health problem in developing countries. The 23.7 Mb draft *E. histolytica* genome contains 9938 predicted genes and a striking abundance of tandemly repeated transferRNA-containing arrays.

The genomes of *E. histolytica* and the amitochondrial protist pathogens *Giardia lamblia* and *Trichomonas vaginalis* share several metabolic adaptations. These include reduced or eliminated mitochondrial metabolic pathways. Indeed, the genome data are consistent with the lack of a mitochondrial genome and enzymes of tricarboxylic acid cycle and mitochondrial electron transport chain. Secondary gene loss and lateral gene transfer, mainly from prokaryotes, seem to have shaped *E. histolytica* metabolism. Moreover, *Theileria parva* and *Theileria annulata* have a higher gene density (4035 and 3792 genes, respectively) and more spliced genes. Despite conserved gene sequences and synteny—3265 orthologous gene pairs between the two species—several species-specific genes were identified. Most genes without orthologues are members of unequally expanded gene families with only a small proportion is present as single copies (34 in *T. annulata* and 60 in *T. parva*). The chromosomes of *T. annulata* and *T. parva* have syntenic regions with only a few inversions of small blocks and no movement of blocks between chromosomes. Short breaks in synteny correspond to gene insertions or deletions and often involve members of large gene families such as the *Tpr* genes (*T. parva* repeat) and their counterparts in *T. annulata*, the *Tar* (*T. annulata* repeat) genes.

Like many parasitic protozoa, both *Theileria* species have tandem arrays of genus-specific, hypervariable gene families that are located in the subtelomeres and are predicted to encode secreted proteins. The overall arrangement of subtelomeric genes is conserved with one (or more) ABC-transporter gene(s) marking the boundaries between subtelomeric gene families and the house-keeping genes. Many *Theileria*-specific protein family members incorporate one or more copies of a polymorphic FAINT (frequently associated in *Theileria*) domain. Over 900 copies of FAINT domain are present in ~166 proteins of both genomes. Like the trypanosomatids, evidence of positive immune selection was found in the macroschizont- and merozoite-stage expressed genes. Also, several candidate genes for host cell transformation have been identified, by assuming that these genes are expressed in macroschizonts and that their products are released into the host cell cytoplasm or expressed on the parasite surface.

Compared with *Plasmodium falciparum*, the metabolism of *Theileria* spp. is streamlined. Several biosynthetic pathways are absent, including those for haem, type II fatty acids, polyamines, and shikimic acid. *Theileria* spp. have lost the ability to salvage purines and have limited ability to interconvert amino acids, but isoprenoid biosynthesis is, however, present. This reduced metabolism suggests substantial dependence on the host cell for many substrates (Hertz-Fowler et al., 2005).

Protozoa represent one of the oldest forms of animal life. They have become adapted to almost all types of environment, the availability of food and water being the most important factors governing their prevalence in different microenvironments. Few protozoa can synthesize their food from inorganic materials and therefore they depend on the

available organic substances such as disintegrating plant or animal material or, relevant here, living microorganisms. Hence, soils that contain high levels of organic matter and bacteria will support an abundance of protozoa. The survival of protozoa under fluctuating environmental conditions is enhanced by the ability to form cysts. Amoebic cysts are resistant to many different environmental stresses such as disinfectants and desiccation. Free-living protozoa are known to occur concurrently with pathogenically important bacteria. These predatory protozoa graze on bacterial biofilms in the environment as a food source. Some bacteria, however, are resistant to killing by protozoa. This intra-amoebic survival of bacterial pathogens and the use of the amoebic cyst to avoid environmental stress and aid dissemination have highlighted the protozoa as the Trojan Horses of the bacterial world. Free-living protozoa have been shown to aid the survival and persistence of numerous pathogenic bacterial species.

The possibility that this might also apply to mycobacterial pathogens is receiving increased attention. Importantly, these relationships between protozoa and bacterial pathogens are highly exploitable and are being used to investigate virulence factors in a broad range of species, including *Legionella pneumophila, L. monocytogenes, Vibrio cholera, Chlamydia pneumoniae, Burkholderia, E. coli*, and methicillin-resistant *S. aureus*. As a laboratory host cell that can differentiate mycobacteria on the basis of virulence, the amoeba provides us with a biologically relevant and robust tool for the investigation of mycobacterial virulence genes, information that is essential to the generation of new treatments and vaccines (Rhodes et al., 2007).

1.3.8. Microscopic Invertebrates

While looking for protozoa, algae, and cyanobacteria in the pond, one invariably encounters large, transparent, complex microorganisms that, to the inexperienced, appear as protozoan. In most instances, these moving "monsters" are "rotifers"; in some cases, they are copepods, daphnis, or any one of the other forms.

All organisms are multicellular with an organ system. If an organ system is present, then these organisms cannot be protists, because the organ system represents tissue differentiation. Collectively, these microscopic forms are designated as *invertebrates* (Fig. 1.28). A few invertebrates such as *Dugesia* and *Hydra* are macroscopic in adult form but they are microscopic when immature. The following phyla are listed according to the degree of complexity, the simplest being the first.

1.3.8.1. Coelenterata

Members of this phylum/family are almost exclusively found in marine habitat. The only common freshwater form is *Hydra*. In addition, there are a few less-common freshwater genera similar to the marine hydroids.

The hydra is very common in ponds and attached to rocks, twigs, or other substrata. Around the mouth at the free end are five tentacles of various lengths, depending on the species. Smaller organisms such as *Daphnia* are grasped by the tentacles and passed on to the mouth. These animals have a digestive cavity that makes up the bulk of the interior. Since no anus is present, undigested remains of the food are expelled through the mouth.

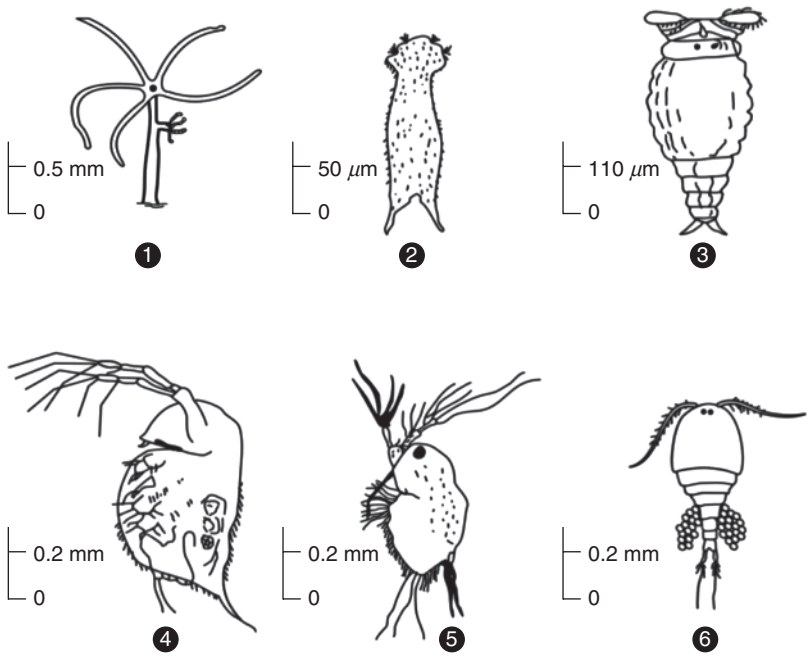

Figure 1.28. Microscopic invertebrates: (1) *Hydra*, (2) *Lepidermella*, (3) *Philodina*, (4) *Daphnia*, (5) *Latonopis*, (6) *Cyclops*.

1.3.8.2. Platyhelminthes

The invertebrates of this phylum are commonly referred to as *flatworms*. The phylum contains two parasitic classes and one free-living organism, the *Turbullaria*. It is the organism that is encountered in freshwater. The characteristics common to all these organisms are dorsoventral flatness, a ciliated epidermis, a ventral mouth, and eyespots on the dorsal surface near the anterior end. As in the coelenterates, undigested food must be ejected through the mouth since no anus is present. Reproduction may be asexual by fission or fragmentation; generally, however, reproduction is sexual, each organism having both male and female reproductive organs. Species identification of the turbellarians is exceedingly difficult and is based, to a greater extent, on the details of the reproductive system.

1.3.8.3. Nematoda

The members of this phylum are the roundworms. They are commonly known as *Nemas* or *nematodes*. They are characteristically round in cross section, have an external cuticle without cilia, lack eyes, and have a tubular digestive system complete with the mouth, intestine, and anus. The males are generally much smaller than the females and have a hooked posterior end. The number of the named species is only a fraction of the total nematodes in existence. Species identification of these invertebrates requires very detailed study of the many minute anatomical features, which requires complete knowledge of anatomy.

1.3.8.4. Aschelminthes

This phylum includes classes Gastrotricha and Rotifera. Most of the members of this phylum are microscopic. This proximity of the nematodes in classification is due to the type of the body cavity pseudocoel that is present in both phyla.

The gastrotrichs are very similar to the ciliated protozoans in size and in habits. The typical gastrotrich is elongate, flexible, forked at the posterior end and is covered with bristles. The digestive system consists of an anterior mouth surrounded by bristles, a pharynx, intestine, and posterior anus. Species identification is based partially on the shape of the head, tail structure and size, and distribution of the spines. Overall length is also an important characteristic of identification. They feed on unicellular algae.

The rotifers are most easily differentiated by the wheel-like arrangement of the cilia at the anterior end and by the presence of chewing pharynx within the body. They are considerably diverse in their food habits; some feed on algae and protozoa, others on juices of the plant cells, and some are parasitic. They play an important role in keeping the water clean. They also serve as food for small worms and crustaceans, being an important part of the link in the food chain of freshwaters.

1.3.8.5. Annelida

This phylum includes three classes: Oligochaeta, Polychaeta, and Hirudinea. Polychaetes are primarily marine and leeches are macroscopic and parasitic. Some oligochaetes are found in marine habitats, but most are found in freshwater and soil. These worms are characterized by body segmentation, bristles on each segment, and an anterior mouth. Although most of the oligochaetes breathe through the skin, some of the aquatic forms possess gills at the posterior end or along the sides of the segments. Most oligochaetes feed on vegetation; some feed on the mud at the bottoms of polluted waters, aiding in purifying such places.

1.3.8.6. Tardigrada

These invertebrates are of uncertain taxonomic position. They appear to be closely related to both Annelids and Arthropods. They are commonly referred to as *water bears*. They have a head, four trunk segments and four pairs of legs. The ends of the legs may have claws, fingers, or disc-like structures. The anterior ends have retractable snouts with teeth. Sexes are separate and females are oviparous. They are primarily herbivorous. Locomotion is by crawling, and not by swimming. During desiccation of their habitat, they contract to form barrel shaped *tuns* and are capable of surviving years of dryness, even in extremes of heat and cold. Widespread distribution is due to dispersal of the tuns by the wind.

1.3.8.7. Arthropoda

This phylum contains most of the known Animalia, almost a million species. The three representatives groups Cladocera, Ostracoda, and Copepoda of the class Crustacaea have in common jointed appendages, an exoskeleton, and gills. The cladocera are represented

by *Daphnia* and latonopsis. They are commonly known as water fleas. All cladocera have a distinct head. The body is covered by a bivalve-like carapace. There is often a distinct cervical notch between the head and body. A compound eye may be present; when present it is movable. They have many appendages; antennules, antennae, mouth parts, and four to six pairs of legs. The ostracods are bivalved crustaceans that are distinguished from minute clams by the absence of lines of growth on the shell. Their bodies are not distinctly segmented. They have seven appendages. The ends terminate with a pair of caudal furca. The copepods such as *Cyclops* and *Canthocamptus* lack the shell-like covering of the ostracods and cladocera.

1.3.9. Microbial Interrelationships

Organisms do not exist alone in nature but in a matrix of other organisms of many species. Thus, interaction is the rule of nature. When one or more types of organisms reciprocate each others effect, it is called interaction. The evidence for such interaction is direct. Population of one species is different in the absence and in the presence of a second species. Interaction between two different biological population can be classified according to whether both population are unaffected by the interaction, one/both population benefit, or one/both population are adversely affected.

A microbial ecosystem represents a delicately balanced population of microorganisms each interacting with and influencing the other members of the population (Fig. 1.29). An understanding of the nature and effects of these interactions is essential to improve the performance of these ecologies, which are important, in such diverse processes as biological waste treatment procedures, water pollution abatement, industrial fermentations, soil microbial association, and human or animal digestive processes. The frequency of microbial interaction may be visualized if one stops for a moment to think of the enormous numbers of microbes. Therefore, while speaking of microbial interactions in a natural habitat such as soil or water, we must realize that the microbes live there in microenvironments as populations of millions or billions and those they intermingle on a scale that is impossible for higher plants and animals. Microbes usually interact in a positive or negative manner. Neutralism is not frequent in the case of this kind of relationship.

Among single population of microbes (microbes of the same species), microbes cooperate at low densities and compete at high cell densities. Beneficial interactions among microbes are facilitated by close physical proximity, for example, biofilms and flocs. Commonly, positive interactions dominate at low population densities and negative interactions dominate at high population densities. Hence, there is an optimal population density for maximal growth rate. The various positive interactions within microbial populations are as follows.

There are many symbiotic relationships among microbes and between microbes and higher organisms. Microorganisms have developed mechanisms to defeat an animal's defense against disease. There is deep symbiosis between some bacteria and their human hosts. Three approaches have been used to visualize the interactions of the world of microscopic organisms: *The first approach* is to isolate the numerous microbial sp., define their biochemical functions, and deduce their activity in a mixed culture of natural environment. *The second approach* seeks to overcome this dilemma by direct observation under microscope. Thus, a series of snapshots of microbial populations, their types, and interactions are taken directly from the soil to the microscope. *The third approach* uses laboratory models of microbial associations, whereby pure cultures are combined in the

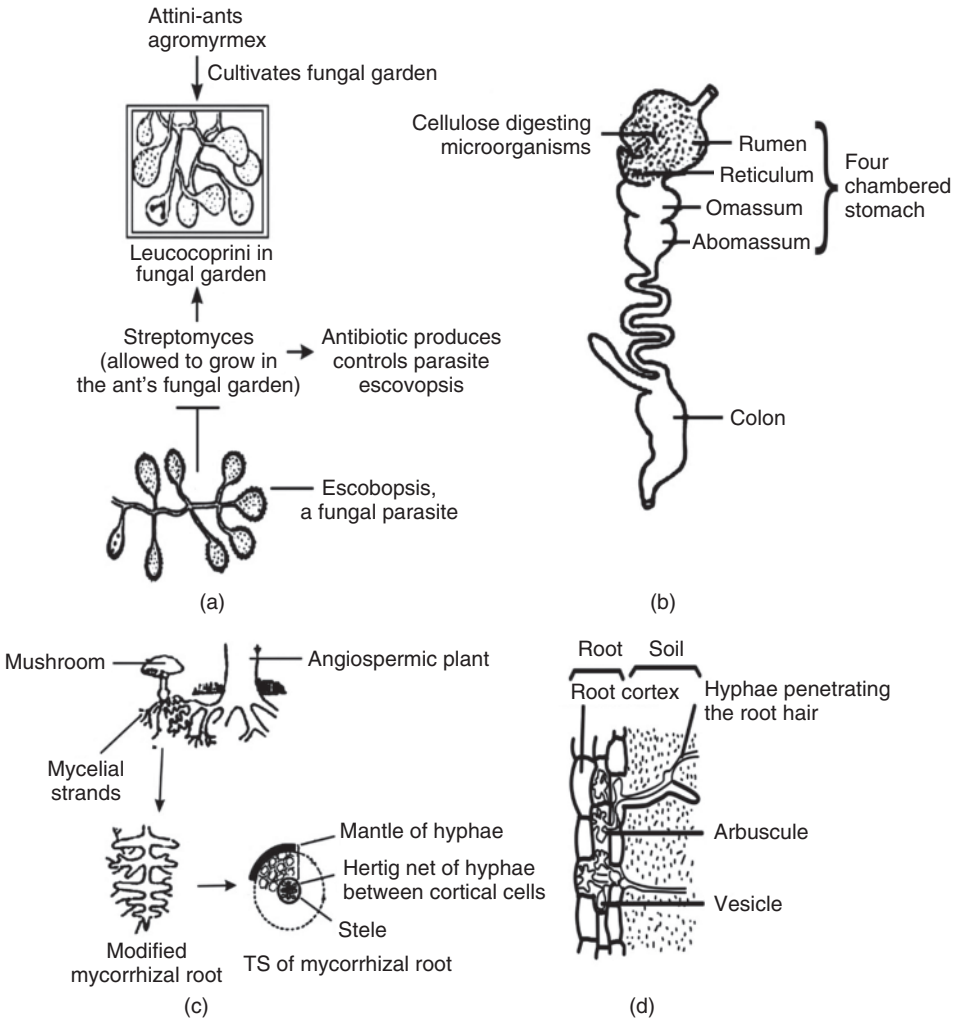

Figure 1.29. Microbial association: (a) schematic diagram showing the use of antibiotic-producing *Streptomyces* by attini ants to control the growth of fungal parasite in their fungal garden; (b) alimentary canal of ruminants; (c) ectotrophic mycorrhiza; (d) VA mycorrhiza.

laboratory under conditions that imitate natural habitat but can be controlled and analyzed. This approach is an excellent method to study interactions between two organisms under varying environment (Weindling and Ingols, 1956).

There are several types of microbial interactions, such as commensalism, inhibition, food competition, predation, parasitism, and synergism, which either singly or in combination may influence the functioning of the microbial ecology. To understand interactions, it is necessary to perform a detailed study of the physiology of the individual predominating microorganism to establish their requirements with respect to such environmental factors as nutrients, temperature, pH, oxidation–reduction potential, removal of waste products, or toxic materials, which may be involved in control processes, and to determine how these factors affect their capabilities. The sum total of this information will

indicate the possible interactions between the microorganisms and will form the basis for conducting experiments either in the laboratory or with mathematical models. Such experiments will lead to an understanding of microbial activities and to the formulation of control measures, often using an alteration of the environmental factors for regulation of the microbial ecologies. Extensive research remains to be done on the microbial interactions to obtain the desired and precise control of these ecological processes (Gall, 1970).

1.3.10. Probiotic Microbes

Probiotics are live microorganisms that confer benefit to the host. They can ameliorate or prevent diseases including antibiotic-associated diarrhea, irritable bowel syndrome, and inflammatory bowel disease. Probiotics are likely to function through enhancement of barrier function, immunomodulation, and competitive adherence to the mucus and epithelium (Ohland and Macnaughton, 2010). Probiotics are bacteria that we eat and they are good for our health. They are found in a number of foods that are readily available in the supermarket, and they taste good. Since probiotic microbes do not cause diseases, there is no such thing as having too much of them. Probiotics are, in most cases, bacteria that are similar to beneficial microorganisms found in the human gut. They are also called *friendly bacteria* or *good bacteria*.

Probiotic bacteria are beneficial bacteria living in the human gut that are now widely used as food additives for their health-promoting effects (Anukam, 2007). These bacteria have coevolved with their human host over millions of years. Their contributions to health and to the development of the host's immune system depend on an intricate web of bacteria–bacteria and bacteria–host relationships that if thrown out of balance will most likely result in a disease.

Probiotics are available in foods and dietary supplements (for example, capsules, tablets, and powders) and in some other forms as well. Examples of foods containing probiotics are yogurt, fermented and unfermented milk, miso, tempeh, and some juices and soy beverages. In probiotic foods and supplements, the bacteria may have been present originally or added during preparation.

Most often, the bacteria come from two groups, *Lactobacillus* or *Bifidobacterium*. Within each group, there are different species (for example, *Lactobacillus acidophilus* and *Bifidobacterium bifidus*), and within each species, different strains (or varieties). A schematic diagram showing the various functions of *Bifidobacterium* are shown in Figure 1.30. A few common probiotics, such as *Saccharomyces boulardii*, are yeasts, which are different from bacteria.

Some probiotic foods date back to ancient times, such as fermented foods and cultured milk products. Interest in probiotics, in general, has been growing. There are several reasons that people are interested in probiotics for health purposes. Researchers are exploring whether probiotics could halt unfriendly microorganism in the first place and/or suppress their growth and activity in conditions such as infectious diarrhea, irritable bowel syndrome, inflammatory bowel disease (e.g., ulcerative colitis and Crohn's disease), infection with *H. pylori* (a bacterium that causes most ulcers and many types of chronic stomach inflammation), tooth decay and periodontal disease, vaginal infections, stomach and respiratory infections that children acquire in day care, and skin infections. Another part of interest in probiotics stems from the fact that there are cells in the digestive tract connected with the immune system (Fig. 1.32).

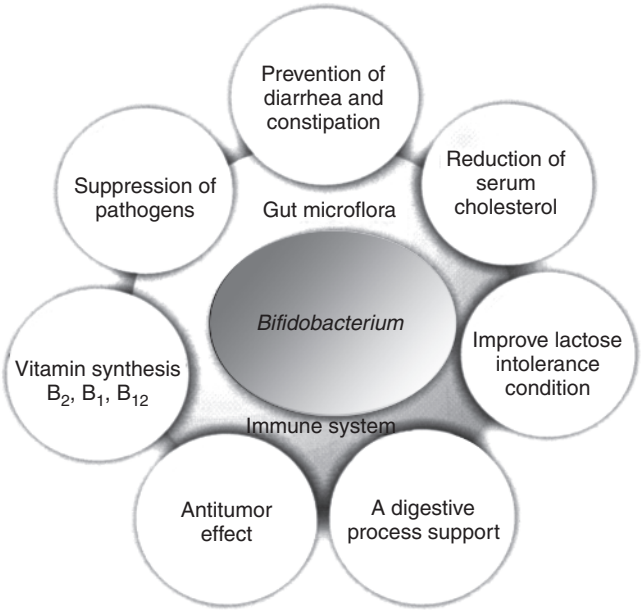

Figure 1.30. Various functions of *Bifidobacterium* as probiotics.

The scientific understanding of probiotics and their potential for preventing and treating diseased conditions are at an early stage, but moving ahead. In general, probiotic side effects, if they occur, tend to be mild and digestive (such as gas or bloating), but in some people more serious effects have also been seen. Probiotics might theoretically cause infections that need to be treated with antibiotics, especially in people with underlying health conditions. They could also cause unhealthy metabolic activities, too much stimulation of the immune system, or gene transfer.

The use of probiotics for prevention and/or treatment of gastrointestinal maladies are becoming increasingly more common within the health-care sector than ever before. The commercial and public interest in live microbes taken as food supplements is equally having a paradigm shift for urogenital health benefits (Kingsley, 2007).

1.4. FUTURE CHALLENGES: METAGENOMICS

Metagenomics enables the DNA from all microbes to be sequenced at once, without any culturing. Such an approach was impossible even a decade ago. As the use of metagenomics has become increasingly common, scientists have had to address the challenge of analyzing an enormous number of genomic sequences (Handelsman, 2004).

Comparing such a huge number of metagenomes is an enormous computational task. But, with metagenomics, job seems shortened. This automated technology revolutionizes the steps needed to acquire an accurately annotated genome. The database allows an overview of the microbial communities and the ability to focus on one metabolic area and detect differences in the proteins being used by the microbes in each environment (Fig. 1.32).

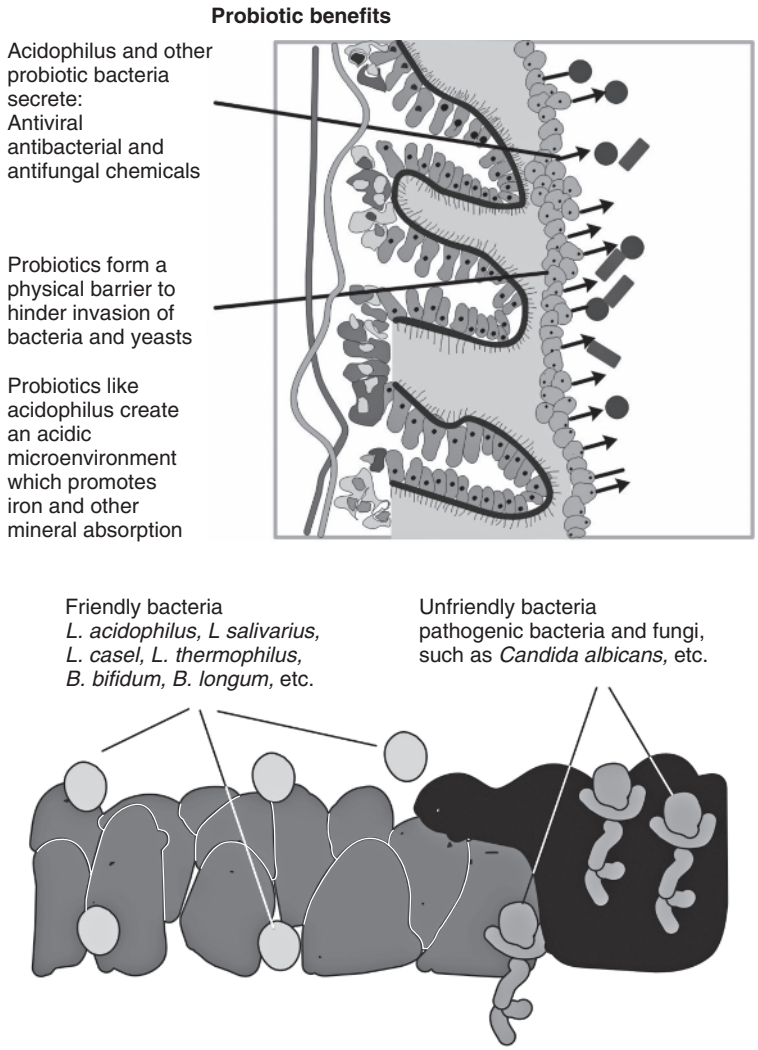

Figure 1.31. Intestinal association of probiotics and their effects.

Comparative metagenomics is a technique that characterizes the DNA content of whole communities of organisms rather than individual species. Statistical analysis of the frequency distribution of 14,585,213 microbial and viral sequences explained the functional potential of nine biomes. In contrast to researchers expectation to find similar behaviors among the metagenomes in every environment, they have distinctive metabolic profiles.

This discovery could lead to innovations in curing viral or bacterial diseases, as well as help to develop new methods of environmental conservation. Metagenomics is a discipline that enables the genomic study of uncultured microorganisms. This evidence was derived from analyses of 16S rRNA gene sequences amplified directly from the environment, an approach that avoided the bias imposed by culturing and led to the discovery

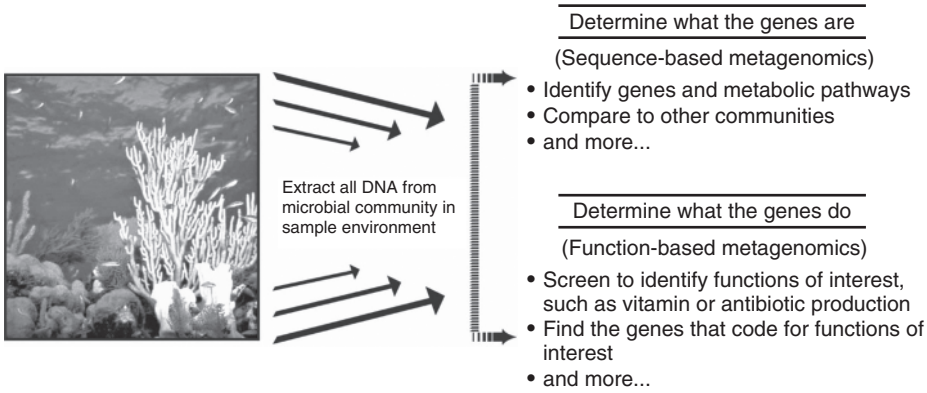

Figure 1.32. The metagenomic process.

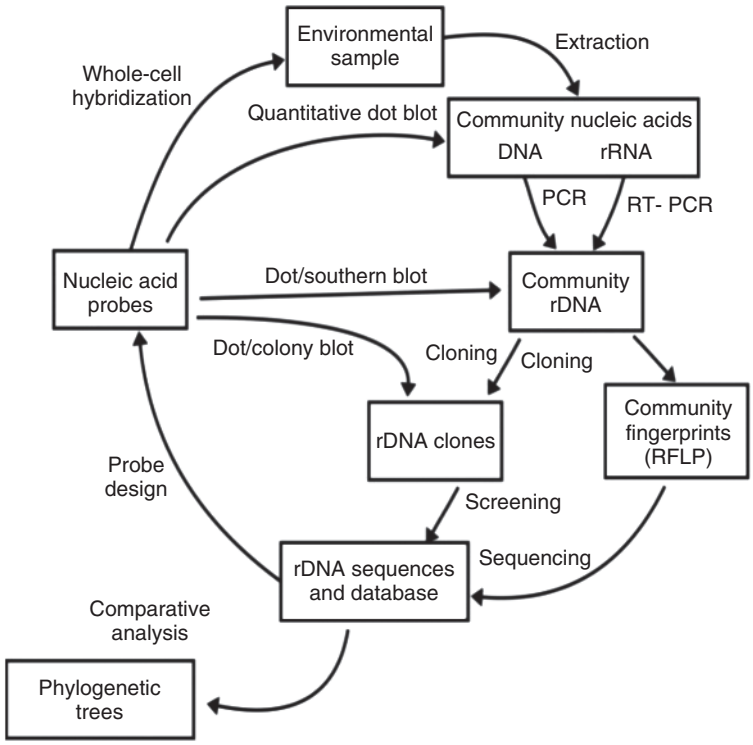

Figure 1.33. Strategies based on rRNA sequences for characterizing microbial communities without cultivation.

of vast new lineages of microbial life (Fig. 1.33). Although the portrait of the microbial world was revolutionized by the analysis of 16S rRNA genes, such studies yielded only a phylogenetic description of community membership, providing little insight into the genetics, physiology, and biochemistry of the members (Handelsman, 2004). Faster, cheaper sequencing technologies and the ability to sequence uncultured microbes sampled

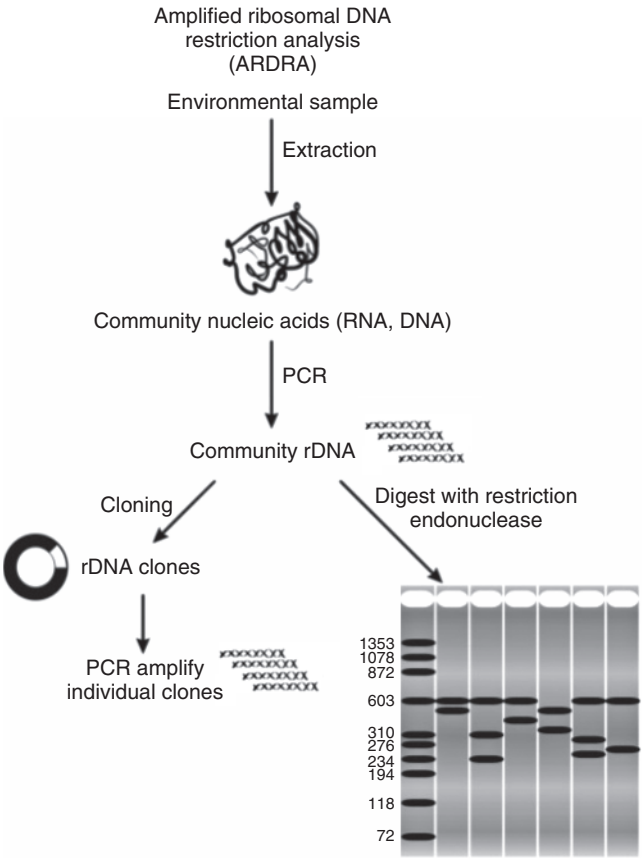

Figure 1.33. (*Continued*).

directly from their habitats are expanding and transforming our view of the microbial world. Distilling meaningful information from the millions of new genomic sequences presents a serious challenge to bioinformaticians. In cultured microbes, the genomic data come from a single clone, making sequence assembly and annotation tractable. In metagenomics, the data come from heterogeneous microbial communities, sometimes containing more than 10,000 species, with the sequence data being noisy and partial. From sampling, to assembly, to gene calling and function prediction, bioinformatics faces new demands in interpreting voluminous, noisy, and often partial sequence data. Although metagenomics is a relative newcomer to science, the past few years have seen an explosion in computational methods applied to metagenomic-based research.

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