6 Phage Ecology

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6.1. INTRODUCTION

Felix d'Herelle, co-discoverer of phage, had a strikingly modern approach to biology. Nearly 100 years ago, he used living organisms to control pests (diarrhea-causing bacteria to halt locust epidemics) and disease (phage therapy of diarrheal diseases). His approaches reflected ecological insights before this branch of biology became an established scientific discipline. In fact, one might have predicted that phage research would become a springboard for studies of microbial ecology. Instead, studies of phage ecology were largely ignored and phage research became the cradle of molecular biology. This turn in the history of biological research is not explained by any critical technical breakthrough, but rather by a number of biographical reasons in the lives of a handful of scientists. The second generation of Western phage researchers concentrated on a few phages from *E. coli*, the workhorse of bacterial genetics, in order to better understand the basic nature of phages and of the phage infection process and use this knowledge to explore fundamental aspects of biology at the molecular level. Phage ecology was not within their conceptual framework. The diversity of phages was better appreciated by medical microbiologists, who used phages for the typing of clinical isolates of bacterial pathogens. However, in that field phages were exclusively used as tools without intrinsic interest in their ecology or molecular characteristics. Consequently, the first monograph on the distribution and behavior of bacterial viruses in the environment appeared only in 1987 (Goyal).

Since the appearance of Goyal's book, the study of phage ecology has fundamentally changed. One drastic reminder of the importance of phage in the ecosystem was the surprising discovery of very large numbers of phage-like particles in the ocean (Bergh et al., 1989). Phage ecology quickly became an intensively investigated branch of marine microbiology, as documented by a recent review listing hundreds of publications, mostly from the last decade (Wommack and Colwell, 2000). Recently, general reviews have appeared on various major aspects of phage ecology (Abedon, 2005; Ashelford et al., 2003; Azam and Worden, 2004; Breitbart et al., 2002; 2003; Chibani-Chennoufi et al. 2004; Paul and Kellogg, 2000; Suttle, 2000b).

Scientists in two fields have developed a particularly keen interest in phage ecology. One is the food industry, where fermentation techniques are used to transform milk, vegetables, or meat into processed foods like cheese, sauerkraut, or salami. This food production relies either on spontaneous fermentation or, in the case of milk, on fermentation initiated by the addition of industrial bacterial starters. Phages that infect these starters are the major cause of fermentation failures in the dairy industry (Chapter 10). The high economic losses associated with phage infection there motivated intense research into phages from lactic acid bacteria, which are the major dairy starter organisms. As the dairy factory is a man-made environment, it did not so much attract the interest of ecologists, but rather that of more technologically oriented microbiologists, focusing on the design of efficient starter rotation systems and the construction of phage-resistant starter cells. In doing that, dairy microbiologists had to investigate the factory ecology of phage infections, leading to large systematic collections of dairy phages. However, these phages were charactarized more by sequence analysis and molecular biology than by classical ecological approaches. The other emerging field is linked to the rekindling of interest

in phage therapy, as discussed in Chapters 13 and 14. The successful application of this approach to the growing problem of antibiotic resistance depends on the availability of large collections of phages and a detailed knowledge of phage-host interactions in different physiological compartments that necessitates sound ecological knowledge of the interactions between the phage, bacteria, and plant or animal host. It is also increasingly extending to interest in potential applications against plant pathogens, bacteria in biofilms, and other complex real-world situations.

6.2. GENERAL PRINCIPLES

6.2.1. PHAGE NUMBERS IN THE NATURAL WORLD

A short 1989 *Nature* paper by a Norwegian group reported 10^7 phage-like particles per ml of coastal and open ocean water, and even 20-fold higher concentrations in a pre-alpine lake (Bergh et al., 1989). Two independent *Nature* papers in the following year confirmed the findings and provided experimental evidence that these viruses limit the primary productivity of cyanobacteria, the major oceanic photosynthetic bacteria (Suttle, 1990; Proctor, 1990). Goyal's 1987 *Phage Ecology* monograph still started the marine chapter with a quote suggesting that phages isolated from the oceans are not indigenous to the marine environment but are transported to the sea by rivers or sewage. Now we know that phage are universally observed in the open and coastal ocean all over the world; in surface water and in great depth (Cochlan, 1993); in ocean ice (Maranger, 1994) and in ocean sediment. Even higher phage concentrations have been detected in marine sediments than in the water columns above them. Counts of up to 10^9 phage particles per ml of sediment were reported by Danovaro and Serresi (2000). Meaningful studies of phage distribution are much more difficult in terrestrial ecosystems, but such techniques as electron microscopy suggest concentrations of the order of 10^7 viruses/gram in soil (Ashelford et al., 2003) and in the feces of ruminants (Furuse, 1987). There seems now to be broad agreement that phage are the most abundant life form on earth; the total number is generally estimated at 10^{30} to 10^{32}. Most of our detailed quantitative data still comes from marine environments, where extensive ongoing mixing makes meaningful sampling possible, but many of the principles and insights also seem applicable to other environments.

In all but the most extreme environments, large numbers of different bacteria and phages are found; there is growing suspicion that phages may represent the largest unexplored reservoir of sequence information in the biosphere. For example, in the case of Chesapeake Bay, Wommack et al. (1999) have estimated that there are about 100–300 phage strains (with many variants of each), infecting 10–50 different bacterial species. Random sequencing of viral DNA from two uncultured 100-liter marine water samples suggested that they contained between 400 and 7000 different phage types (Breitbart et al., 2002); a similar analysis shows that the human gut also contains hundreds of different phage genotypes (Breitbart et al., 2003).

Marine phage biologists have developed precise data about *in situ* burst sizes (Borsheim, 1993), since this figure is essential for calculations of virus production in the given environment and the level of virus-mediated mortality of bacterioplankton;

such determinations have not generally been possible in other natural habitats. The burst size in the environment is generally smaller than that determined in the laboratory by one-step growth experiments, reflecting the smaller size of bacteria in most natural settings (Robertson and Button, 1989; Weinbauer and Peduzzi, 1994). The nutrient level and temperature of the water sample and the morphology of the host were the most important determinants of the burst size. In situ burst-size determination is mostly done by microscopic observation of virus particles within bacterial cells (Hennes, 1995; Weinbauer et al., 1993). Alternatively, the *in situ* burst size is calculated by balancing viral production with viral decay (Suttle and Chan, 1994), assuming that if the phage concentrations are relatively stable over a given (short) time period, then the rates of phage production and phage decay must be equal. This lets one look at specific viable phage infecting a particular host. Quantitative data on virus decay are important for other fields of research as they provide a measure of the tenacity of a virus in the environment. Such information is crucial for such diverse questions as public health evaluations of viral contamination of water samples, source tracking, the persistence of phages in industrial environments, and the half-life of therapeutic phages.

6.2.2.   DYNAMIC PHAGE-HOST RELATIONSHIPS

Major fluctuations in phage and host numbers are observed over various periods. For example, there is substantial seasonality in marine phage titers (Bratbak, 1990; Hennes, 1995; Mathias, 1995; Cochran and Paul, 1998). The levels are often 10-fold lower in the winter than in the summer months, even though the bacterial densities do not appear to fluctuate by a factor of more than 2–3, with no seasonal trend; the reasons behind this apparent uncoupling of bacterial and phage populations are not yet clear, but one explanation could be increased induction of prophages in the summer months due to higher sunlight exposure. Another possibility is that phage production itself is much more temperature sensitive, in general, than is bacterial growth. At the same time, the UV in sunlight causes up to 5% loss in viable phage per hour for surface water due to production of thymine dimers (Wommack, et al., 1996); this causes far more loss in summer than in winter at more polar latitudes. A strong shift in balance was seen over 6 months between the two major phage types infecting the rhizosphere of sugar beets: from *Siphoviridae* with long latent period and big burst sizes to *Podoviridae* with short latent periods and small burst sizes, apparently reflecting changes with season in the availability and physiological state of the host bacteria and plants (Ashelford et al., 1999). Variability was also shown over shorter time periods, in one marine study even over half-hour time intervals, demonstrating a highly dynamic relationship between phages and their hosts (Bratbak, 1996).

As discussed in Chapters 3 and 7 and by Abedon (2005), the phage lytic life cycle has at least 4 key steps that are very relevant to phage ecology:

1. An extracellular search that is limited by diffusion rates and thus dependent on host concentration.
2. A phage adsorption step that combines reversible phage binding, irreversible attachment, and genome transfer into the host, which typically occurs

rapidly following productive collision between a phage particle and a phage-susceptible bacterium.
3. An infection step, during which host physiology is appropriately restructured, the phage genome is replicated, and phage particles are assembled.
4. For *temperate* phages, an indefinite period of *lysogeny* may be inserted, during which the phage is inserted in the host genome and replicates with it or replicates synchronously as a plasmid and the genes responsible for lytic growth are repressed, followed by a step in which phage progeny are released from the infected bacterium; except for filamentous phages, this process involves cell lysis, which usually is carefully timed.

The attachment and lysis steps are generally rapid. Thus, most of the time the phage is either:

1. Free during the extracellular search,
2. Trapped in some compartment where no bacteria are readily available, such as bound to relatively inert particulate matter,
3. Actively infecting a bacterium, leading eventually to cell lysis or phage secretion, or
4. Existing as a prophage

For the first two, the phage may become inactivated through capsid or genome damage, but it also may remain infectious for many years, depending on environmental conditions. The lytic infection phase seldom lasts more than a few minutes to a day, though there are conditions like the one we call "hibernation" in which the phage genome remains benignly inside until nutrients become available and the host resumes more active growth. For virulent phages like T4, this differs markedly from lysogeny, though, in that the phage genome then takes over and the only possible outcome is host-cell death, accompanied by eventual phage production if nutrients become available, as discussed in 2.4; no colonies are formed.

Our understanding of the phage infection process comes primarily from experiments in which the laboratory researcher mixes a single phage strain with a single bacterial strain at about 10^8 cells/ml. In contrast, in near coastal water, for example, bacterioplankton concentrations are typically 10^6 cells/ml and the population normally consists of 100 different bacterial host species, yielding a mere 10^4 cells/ml for the average host species (Murray and Jackson, 1992). Is this enough to maintain an infection cycle? The answer is apparently yes, since no marine water samples devoid of phage were ever reported, and enrichment procedures generally permit the isolation of phage against any given host from the particular ecosystem. Yet phage replication is clearly sensitive to effective cell concentration. Laboratory experiments with T4 and *Bacillus* and *Staphylococcus* phages showed no phage production until the host cell concentration reached 10^4 cells/ml (Wiggins and Alexander, 1985). However, studies using *Pseudomonas* phages (Kokjohn et al., 1991) showed evidence of lytic infection at cell concentrations as low as 10^2 cells/ml. In some studies in natural marine environments, no intracellular phage were observed when the number of rod-shaped bacteria fell below 10^5 cells/ ml (Steward et al., 1992;

Weinbauer and Peduzzi, 1994). However, some marine viruses replicated efficiently down to 10^3 specific host cells/ml (Suttle and Chan, 1994; 1993). The ups and downs of host cell concentration over a time series allowed the approximation that cyanophage replication still occurred when the host cell concentration fell to 10^2 cells/ml (Waterbury, 1993).

Such theoretical concerns related to the reproduction of virulent phages at low host densities led to the hypothesis that temperate phages should outnumber virulent phages in the ocean, since the production of temperate phages is independent of host cell density. It has been proposed that lysogeny becomes the preferred strategy when the cell density falls below the lower limit necessary for maintenance of the phage density by repeated cycles of lytic infections (Stewart and Levin, 1984). Lysogens might out-compete the non-lysogenic congeners by the selective advantage conferred by lysogenic conversion genes contributed by many temperate phages. Some of these are relatively universal, such as immunity functions and superinfection-exclusion genes. Other prophages contribute genes that make the lysogen competitive under special ecological situations, such as the serum resistance conferred to the lysogen by the phage lambda *bor* gene under blood growth of *E. coli*. This phenomenon is very marked in lysogenic bacterial pathogens, where many virulence factors are encoded by prophages. However, even laboratory phages like P1, P2, lambda, and Mu led to higher metabolic activity and faster and longer growth than seen in non-lysogens (Edlin et al., 1975; Lin et al., 1977). With this selective advantage even under laboratory conditions and the intrinsic difficulties with the lytic life style, the prediction is a high concentration of lysogens in the oceans. Indeed, two marine surveys revealed 40% mitomycin-C-inducible cells; similar proportions of lysogens were identified in *Pseudomonas* colonies from lakes (Ogunseitan et al., 1992). The surveys showed the trend for lysogeny to be more prevalent in oligotrophic environments (Jiang, 1994; 1997). This observation fits with theory since this setting is dominated by low densities of slow-growing bacteria. However, other data contradict this explanation. Surveys in estuarine waters showed a seasonal development of lysogeny with highs in the summer months when eutrophic conditions were prevalent and lows in the winter months when cells were at their minimum (Cochran and Paul, 1998). There are further contradictions of expectations. First, spontaneous induction of prophages is generally low (10^{-2} to 10^{-5} phage per bacterium per generation) (Stewart and Levin, 1984). This release can only account for <1% of the phage concentrations in the ocean (Jiang, 1997). Second, large phage surveys in the North Sea revealed that only 10% of the phage isolates are temperate (Moebus, 1983).

Paul and Kellogg (2000) extensively explored the available research related to the frequency of lysogeny in natural environments, the factors that can induce such lysogens, and the roles of phages in bacterial genetic exchange in various ecosystems. A variety of approaches have indicated that lysogeny and even polylysogeny are common; the various microbial genome projects to date have confirmed this, with at least half of the sequenced bacteria carrying prophages, and prophages or defective prophages are responsible for many differences between isolates of the same species. The extent of lysogeny varies between different kinds of bacteria; for example, nearly 100% of naturally occurring *Pseudomonas* are lysogenic. Prophages could be

induced from most bacteria in eutrophic lakes and estuaries, while induction was far less common in offshore and northern-lake environments. The degree to which this reflects prophage presence vs. metabolic state is not clear. Inducibility of lysis and phage release by means of mitomycin C or UV are the most common criteria for the presence of lysogeny; however, the two may not induce the same prophages, and many prophages are not induced by either of them. Other inducing agents that have been explored on natural isolates include sunlight, temperature, pressure, poly-nuclear aromatic hydrocarbons (PAHs), fuel oil, and trichloroethylene. PAHs, a PCB mixture, and Arochlor 1248 were the most efficient agents, giving effective induction of prophages in 75% of the tested samples, vs. 50% for 254-nm UV radiation and for mitomycin C. Raising the temperature to as little as 30 degrees for 30 min could induce lysis; Paul and Kellogg suggest that this may help explain the 10-fold summer increase in free phage. Induction also generally works less well when the host cells are at a lower metabolic state, since most inducing agents act on replicating DNA.

In summary, it appears that a key aspect of the high prevalence of virulent phages in the oceans is that the numbers of any given phage-host pair are constantly fluctuating in any natural setting, probably coupled with the continual mixing and astronomical total numbers involved. Phage replicate most rapidly on the most abundant, fastest-growing host population in a given setting at a given time, where new hosts are found most rapidly, thus, for example, terminating microbial blooms (cf. Hennes and Simon, 1995). Various approaches in different aquatic environments suggest that about 15% of the bacterioplankton are lysed by phages daily, leading to the release of nutrients important to marine ecosystems. The fact that most phage will not replicate to a significant degree at host concentrations below 10^3–10^5 per ml assures the maintenance of microbial diversity despite the presence of phages that can infect each potential host. A high fraction of the bacteria in the oceans, as in many other habitats, also harbour one or more prophages, but free temperate phages do not generally contribute substantially to the high concentrations of phages observed in both marine and terrestrial environments.

6.2.3. CO-EVOLUTION OF PHAGES AND THEIR HOST BACTERIA

Classical experiments with *E. coli* and its phages generally showed a rapid outgrowth of phage-resistant bacterial strains. The coexistence of susceptible bacteria and their corresponding phages in the environment was thus a somewhat surprising observa-tion. Further classical experiments showed a co-evolution, with bacteria and their phages involved in a continuous cycle of resistance and counter-resistance mutations. Theoretical ecologists pointed to an asymmetry in this relationship, since receptor mutations arise more easily in bacteria than anti-receptor mutations in phages (Lenski 1984; 1985). Since there are far more phages than bacteria in many ecological settings, phages may balance these differences out. The development of phage-resistant cyanobacteria was demonstrated in the field (Waterbury, 1993). Other researchers argued that virulent phages might survive due to the maintenance of sensitive parental strains in the population if the parental strains have a slight growth advantage. Most discussions of these issues use the terms *resistant* and *sensitive* as if they are absolutes. In nature, one actually sees many cases in which a particular

host can be infected by a given phage, but with low efficiency; this can also lead to co-survival of phage and host. For example, in spot testing nearly 100 phages against a battery of hosts, 10 were identified as able to infect *E. coli* O157; however, the EOP turned out to be only about 10^{-4} for most of them (Kutter lab, unpublished). Only RB69 plated as efficiently on O157 as on B or K12. Parameters affecting the efficiency of plating are discussed in the Appendix, section A.4.1.2.

6.2.4. EFFECTS OF HOST PHYSIOLOGY AND NUTRITIONAL STATUS

The normal state of a marine bacterium was predicted to correspond to the nutritional state of a laboratory bacterium under stationary-phase conditions (Kolter et al., 1993). While gram-negative bacteria do not sporulate, as do some gram-positive bacteria, they do undergo a variety of metabolic and structural changes in stationary-phase conditions that contribute to long-term survival in hostile environments. These are mediated by a new sigma factor, σ^s, which controls at least 30 genes that are expressed during starvation and at the transition into stationary phase. *RpoS* mutants survive much less well under laboratory conditions of carbon or nitrogen starvation and fail to develop starvation-mediated cross protection to oxidative, osmotic, and heat stresses (McCann et al., 1991). From laboratory studies, it has been widely accepted that most phages cannot productively infect stationary-phase bacteria, leading to great surprise at the observed phage concentration levels in the ocean and illustrating gaps in our knowledge when we try to transfer our experiences from laboratory phage-host interactions to the ecological situation.

Clearly part of the problem was the limited number of phage-host systems on which the accepted model had been based. Woods (1976) had actually found that *Pseudomonas* phages could infect starved host cells maintained for 40 days in natural riverine conditions. Under these conditions, the latent period was lengthened and the burst size greatly reduced when compared to logarithmic-phase infection. Schrader et al. (1997) showed that coliphage T7 and the three *Pseudomonas* phages he tested could replicate well in cells that were starved or had entered stationary phase; the variety of patterns seen emphasizes the importance of avoiding generalizations. Phage ACQ could even infect *P. aeruginosa* maintained in starvation conditions for 5 years; the burst size was reduced whether the starvation was short or long, and the latent period was extended severalfold. For T7, the latent period was also lengthened severalfold, but, interestingly, the burst size *increased* from about 50 to 450 when the host had been starved for 24 hours. T4 cannot produce a burst in stationary-phase cells. However, T4 can enter and persist in long-term stationary-phase cells, but as long as the stationary-phase sigma factor is present, the infection process is suspended at an early stage. Whereas T4 normally blocks all host-gene transcription and translation within minutes, it enters what we call "hibernation mode" and produces a stable infective center in these starved cells (Kutter lab, unpublished results; Fig. 6.1); when nutrients become available, the usual host outgrowth proteins are produced, but then the phage program takes over, all further host protein synthesis is blocked, and progeny phage rather than colonies are produced after resumption of cell growth. This phenomenon could help explain the persistence of many virulent phages within populations of non-growing cells.
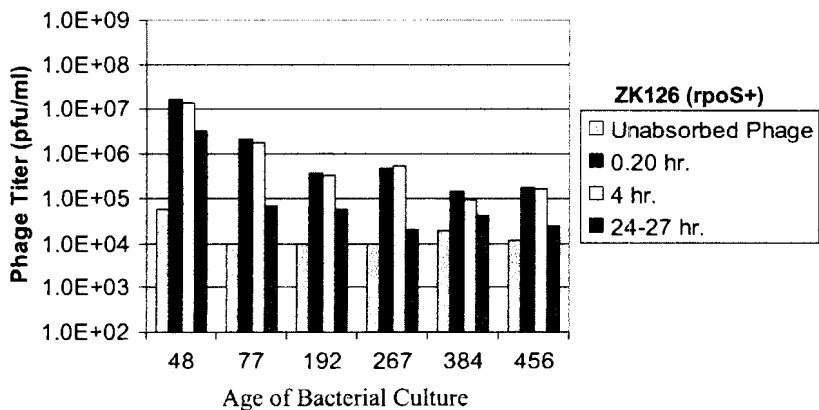
FIGURE 6.1 Ability of bacteriophage T4 to survive in stationary-phase *E. coli* for extended periods and still respond to form an infective-center plaque when transferred to nutrient-rich plates. After growth in TSB for varying times (expressed in hours), 10^7 phage/ml were added to samples of the culture. This ability to form stable infective centers in starved cells depended on the presence of the stationary-phase sigma factor, rpoS; in otherwise-isogenic rpoS cells, few infective centers were seen even at 4 hours (Kutter, 2001).

Complementary observations have been made in *sporulating* bacteria, illustrating some of the complexities of phage-host interactions under growth-limited conditions in the wild. For example, phages φe and φ29 are lytic when infecting growing *B. subtilis*. However, each phage shows a narrow window during sporulation when it can become entrapped in the spore and persist in a quiescent state which allows later germination of the spore followed by formation of an infective center rather than a bacterial colony (Sonenshein and Roscoe, 1969). Also, the complex life cycle of the gram-negative bacterium *Myxococcus xanthus* includes the formation of fruiting bodies containing relatively stable myxospores. Bacteriophage MX-1 is virulent on exponential-phase cells but will not bind to myxospores (Burchard and Dworkin, 1966). For an hour after the transition has been induced, MX-1 still binds and injects its DNA but the phage genome becomes trapped in a persistent state; again in this case, germination begins normally upon nutrient addition, but within an hour the phage program takes over (Burchard and Voelz, 1972).

6.2.5. BIOFILMS

Most studies of the phage infection process have been carried out with bacteria suspended free in liquid culture. However, at interfaces between solid surfaces and aqueous environments, bacteria frequently aggregate to form complex attached communities called *biofilms* (Fig. 6.2). Such biofilms are widespread and pervasive— on river rocks, in pipes, industrial equipment and medical implants, lining the colon, as dental plaque, in the lungs of cystic fibrosis patients (cf. Costerton, 1999). Microorganisms undergo profound phenotypic developmental changes during the
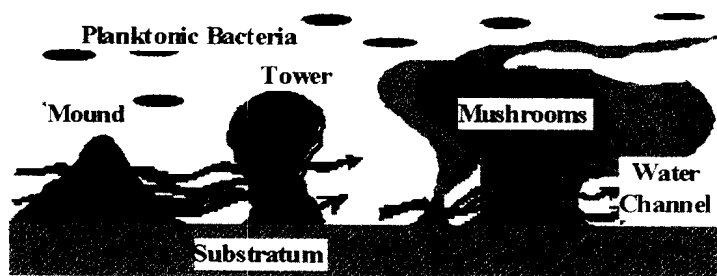
FIG 6.2 Complex structures and water channels proposed for biofilm architecture. Drawn by Kalai Mathee.

transition from free-floating planktonic to biofilm growth (O'Toole et al., 2000). Due to these inherent physiological changes, their slow growth rates and the fact that the cells are embedded in an extensive exopolysaccharide matrix with heterogeneous spatial distribution, cells living in biofilms tend to be at least 10-fold more resistant than planktonic cells to most antibiotics and other antimicrobial treatments (Anwar et al., 1992; Bagge et al., 2004).

Interest is growing in the natural roles of bacteriophages in modulating biofilms and, particularly, in the potential for their use in controlling biofilms in a variety of settings. Adams and Park (1956) first explored the properties of a phage polysaccharide depolymerase, while Lindberg (1977) reported EM observation of these enzymes as spikes attached to the phage baseplate. Here, the capsular material acts as a secondary receptor to which the phage can bind, degrading the polymer until it can reach its outer-membrane receptor and infect the cell (see Chapter 7, Fig. 7.4). In exploring the role of cell death in *Pseudomonas* biofilm development, Webb (1996) found evidence that an induced *prophage* actually played a significant role in the process. They found that after a few days up to 50% of the microcolony structures within biofilms formed by various bacteria showed areas of killing and lysis in their centers, sculpting internal structures and releasing free-swimming bacteria; this was paralleled by the release into the medium of a temperate phage that seemed to be responsible for this process.

Studies have been carried out of phage interactions with biofilms involving such bacteria as *Pseudomonas aeruginosa, E. coli, Listeria monocytogenes, Enterobacter agglomerans*, and *Staphylococcus aureus*. Doolittle et al. (1995; 1996) demonstrated lytic infection of *E. coli* biofilms by bacteriophage T4 and used fluorescent probes to track the interactions of the phage with the biofilms. Hughes (1998), exploring biofilm bacteria from a food processing factory, showed that *E. agglomerans* phage SF153b has a polysaccharide depolymerase that can disrupt biofilms through exopolysaccharide (EPS) degradation even when a phage mutation blocks cell infection and lysis. They partially purified the enzyme, an endoglycanohydrolase, and confirmed that it could work in isolation and was specific for the *Enterobacter* EPS: it could not attack similar biofilms formed by *Serratia*. As demonstrated by Hanlon et al. (2001), appropriate phages could diffuse through alginate gels and produce a 2-log reduction in bacterial numbers in 20-day-old *P. aeruginosa* biofilms, reducing

the viscosity by as much as 40% despite the presence of EPS. Corbin et al. (2001) used a chemostat coupled to a modified Robbins chamber and scanning confocal microscopy and saw clear T4 effects on glucose-limited biofilms, at least at very high multiplicities of infection. Here and in several of the previous cases, there was no evidence for involvement of a degradative enzyme and it is not clear how the phages got through the EPS layer to reach their receptors. McLean et al. (2001) have explored useful techniques for studying the parameters affecting biofilm growth and phage-biofilm interaction.

6.3. MARINE PHAGE ECOLOGY

6.3.1. MARINE PHAGE IMPACTS ON THE FOOD WEB

A number of parameters of the phage-bacterium interaction have been determined in quantitative detail for marine phages, making them currently the best-characterized phage ecology system. The production and distribution of marine viruses is, not surprisingly, determined by the productivity and density of the host populations (Boehme, 1993; Jiang, 1994; Weinbauer, 1995). The usual virus-to-bacterium ratio falls between 3 and 10 and depends clearly on the nutrient level: bacterioplankton produce more phages under environmental conditions favouring fast bacterial growth and productivity (Hara, 1996; Maranger, 1994; Steward, 1996). The quantitative relationships led to numerical calculations of the degree of bacterial mortality caused by marine phages (Binder, 1999) and mathematical modeling of the energy flow in marine food webs. Phage predation of marine bacteria now enters into models of global biogeochemical cycling of carbon (Proctor, 1990). Bacterial and algal viruses are established members of the microbial loop in the oceans with profound effects on the cycling of carbon and nitrogen and on the marine food web (Fuhrman, 1999). The change from discovery of their existence to such a prominent place in ecological modelling could not be more dramatic.

When a bacterium is lysed by phage infection, probably 99% of the cell contents enter the dissolved organic matter pool (Fuhrman, 1992). Phage are thus efficient drivers in the biomass-to-dissolved-organic-matter conversion. They are important sources of bacterial mortality in the sea, along with bacterial grazing by zooplankton. The result of phage lysis of marine bacteria is, paradoxically, a stimulation of bacterial growth. The rationale is the following: The presence of phage stimulates the growth of bacteria in comparison to a situation where phages are lacking. Predation of bacteria by protists results in the transfer of bacterial biomass into the next layer of the food web with no feedback (in the literal sense) to bacteria. Phage lysis, in contrast, releases nutrients from the lysed cells that become available to the bacterial community (Middelboe et al., 1996). In one model, viral lysis causes a net loss of 25% in nanozooplankton production (Fuhrman and Suttle, 1993). To complicate the matter further, some protists like dinoflagellates also prey on viral particles.

Substantial theoretical and experimental research efforts were undertaken to determine the quantitative degree of virus-mediated bacterial mortality and to assess the ratio of phage lysis versus grazing by higher organisms. Not surprisingly, the

greatest impact of phage lysis was in oligotrophic environments. Furthermore, the ratio of lysis versus grazing changed with water depth both in the ocean and in lakes (Steward, 1996; Weinbauer and Hoefle, 1998). To summarize a large body of literature, different approaches in different environments yielded a remarkably stable rate of virus-mediated bacterioplankton mortality of about 15% per day (Suttle, 1994). The rate seems to be higher for heterotrophic bacteria than for the autotrophic cyanobacteria (Suttle, 1994). Even if these figures seem to suggest only a modest effect of phages on bacteria, a 15% bacterial mortality can still have a profound effect on the relative proportions of different species or strains in a community. Models of virioplankton that control host community diversity have been developed. For a eutrophic estuarine environment, the basic numbers were approximated as follows: bacteria at a density of 10^6/ml with about 50 different species and viruses with a concentration of 10^7/ml and 200 different strains (Wommack et al., 1999). One concept is that of "killing the winner populations," i.e. phage expand on the fastest-growing host population in the given ecological setting (Thingstad and Lignell, 1997). The epidemic ceases when the diminished host population no longer supports efficient phage replication. Blooms have been observed where up to 80% of the total bacterial population is represented by a single bacterioplankton strain, although lower peak levels are more frequent. There is strong indirect evidence that some bloom collapse is mediated by viral lysis. The most convincing data are from the Lake of Constance, where transient increases in bacterial abundance were closely followed by peaks in the frequency of infected bacteria and free phage (Hennes, 1995).

Phage infection in the ocean leads to better retention of nutrients in the euphotic zone because more organic material remains in non-sinkable bacterial form (Murray and Eldridge, 1994; Thingstad et al., 1993). In contrast, lesser phage infection allows a transfer of organic material upwards in the food chain, into organisms that eventually either sink themselves or are compacted in the fecal pellets of organisms with guts (Fuhrman, 1992). These processes transport organic masses from the euphotic zone to the deep sea. They have substantial impact on global climate models via CO_2 fixation from the atmosphere into marine biomass and eventual transfer to the ocean sediment, with the net result of a reduction of this important greenhouse gas (Wilhelm and Suttle, 1999); phage infection helps counteract this. Marine viruses may have an additional effect on the shaping of the global climate by inducing the release of dimethyl sulfide (DMS) from lysed phytoplankton (Malin et al., 1998). DMS is a gas that nucleates cloud formation and thus affects the radiative properties of the atmosphere.

As discussed in section 6.2.1, much quantitation has been carried out in the marine environment, but extrapolation of data from the marine field to others is often problematic. Ocean water often contains substances that protect viruses from inactivation, such as adsorption to clay particles (LaBelle and Gerba, 1982; Smith et al., 1978), as well as heat-labile uncharacterized virucidal substances that show geographical variation (Suttle and Chen, 1992). In principle, two processes must be differentiated: the destruction of phage particles and the loss of infectivity. There is a consensus that unattenuated sunlight is the dominant factor controlling the decay of viral infectivity in surface waters (Garza and Suttle, 1998). Inactivation

by sunlight was significant down to a depth of 200 m. UV-A (320 to 400 nm) has the greatest impact (Murray and Jackson, 1993). UV-mediated dimer formation of adjacent pyrimidines was the principal photodamage (Wilhelm et al., 1998). However, host- and phage-encoded photorepair systems could still recover some of the lost infectivity (Bernstein, 1981). Phages with smaller capsid size turned out to be more sensitive than phages with capsids >60 nm (Heldal and Bratbak, 1991; Mathias, 1995). One to five percent infectivity loss per hour was the average, and no marked differences were observed between marine phages and reference laboratory phages (Wommack et al., 1996); under surface light conditions, a one-log loss of infectivity was observed over a day of natural sunlight exposure. Differences were observed between distinct phage isolates infecting the same host, demonstrating that environmental persistence is a trait particular to a given phage strain. Non-native phages experienced a greater sunlight inactivation than native phages, suggesting adaptation of phages to local conditions (Noble and Fuhrman, 1997).

6.3.2. MARINE PHAGE CHARACTERIZATION

6.3.2.1. Cyanophages

Cyanobacteria are among the most important primary producers and nitrogen fixers on earth, responsible for much of the primary production in oceans, lakes, and other aqueous environments. They are ubiquitous, found in extreme environments from hot springs to polar lakes as well as in ponds for waste stabilization and for raising fish. They can be differentiated into marine and freshwater forms, into those using phycoerythrin vs. those using phycocyanin as their primary photosynthetic pigment, and into those that are unicellular vs. those that grow as filaments. While they are clearly bacteria, their ecological roles are more closely tied to those of eukaryotic algae than to those of heterotrophic bacteria (Suttle, 2000a; Suttle, 2000b).

Cyanophages infecting filamentous freshwater cyanobacteria were first isolated by Safferman and Morris (1963), and stimulated the hope of using such viruses to control cyanobacterial blooms. Phage infecting filamentous cyanobacteria generally cause rapid invagination and destruction of the host's photosynthetic membranes, whereas such destruction is only seen very late in the infection cycle with those infecting unicellular cyanobacteria, for which successful infection seems to depend on ongoing photosynthesis.

Demuth et al. (1993) reported that nearly all of the phages they saw in Lake Pluβsee had tails, the majority being *Siphoviridae*; in those studies, the phage levels were so high that they simply floated the TEM grids on the samples and let the phages adsorb. Half of the phages seen in Chesapeake Bay by Wommack et al. (1992) also had tails. *Myoviridae* are the most common characterized from marine waters and are also often isolated from fresh-water species. Five *Synechoccus* cyanophages isolated by Wilson et al. (1993) included two myoviruses and one siphovirus. Head proteins of one group of marine phages even have clear sequence relationships to T4-like coliphages, as discussed below. The similarities are particularly interesting since cyanobacteria diverged from other bacteria billions of years ago.

6.3.2.2.   Genomic Analysis of Marine Phages

Various genomics approaches have given new insights into the field of phage ecology. A large-scale random sequencing effort of two uncultured marine water samples demonstrated that 65% of the sequences lacked matches to the database (Breitbart et al., 2002). The database hits were mostly with viruses, covering all major families of tailed phages and some algal viruses. A careful statistical analysis of the sample revealed between 400 and 7000 different viral types in the two 100-litre samples, with the most abundant type representing 3% of the total viral population. Over 200 *Vibrio parahaemolyticus* phages were isolated from various locations and seasonal periods in Florida and Hawaii (Paul and Kellogg, 2000). All observed isolates were *Myoviridae* and shared some genetic determinants, giving 83%–100% identity for one sequenced 500 bp region, but on the basis of restriction patterns they could be divided into at least 7 groups for the Florida isolates, one of which was consistently dominant (71%), plus a pair of Hawaii isolates.

In striking contrast to the careful ecological work performed with marine viruses, only a handful of marine phages have actually been sequenced. However, these few examples delivered surprises. A *Pseudoalteromonas* phage with a 10 kb genome became the type phage of a new family, called *Corticoviridae*, of lipid-containing phages. Cyanophage P60 and phage SIO1, infecting the marine heterotroph *Roseobacter,* resembled coliphage T7 closely in their genome organization. Three *Synechoccus* cyanophages were found to share distant head-gene sequence relationships with coliphage T4 (Fuller et al., 1998). Zhong et al. (2002) then designed primers to amplify capsid assembly protein gp20 from both isolated marine cyanophages and natural virus communities and looked at 114 different gp20 homologues. He found these cyanophages to be a highly diverse family, with 65%–96% sequence similarity among the cyanophage gp20's (and 50%–55% similarity with T4). They fall into 9 different phylogenetic groups (Fig. 6.3), with up to 6 clusters and 29 genotypes found in a single sample.

6.3.3.   Marine Phage Host Specificity and Horizontal Gene Transfer

In the large majority of the phages investigated in the laboratory, host species specificity is the rule (Ackermann, 1987); phages generally also display strain specificity within a host species due to using a variety of different receptors. Phages with broad host ranges have been described, but they are the exceptions. There was some expectation that marine phages might show broader host range to facilitate their multiplication at the frequently low host densities seen in ocean environments, as discussed above. However, the results to date indicate that most of them are host-species specific; many also demonstrate strain-specificity (Baross et al., 1978a; Bigby and Kropinski, 1989; Koga et al., 1982; Moebus, 1992). Striking host range differences were seen between phages recovered east or west from the Azores islands in the Atlantic Ocean (Moebus and Nattkemper, 1981). Broad host range was more prevalent in cyanophages, but Hennes (1995) demonstrated that fluorescence-labelled cyanophages attached specifically only to their known host and not to other
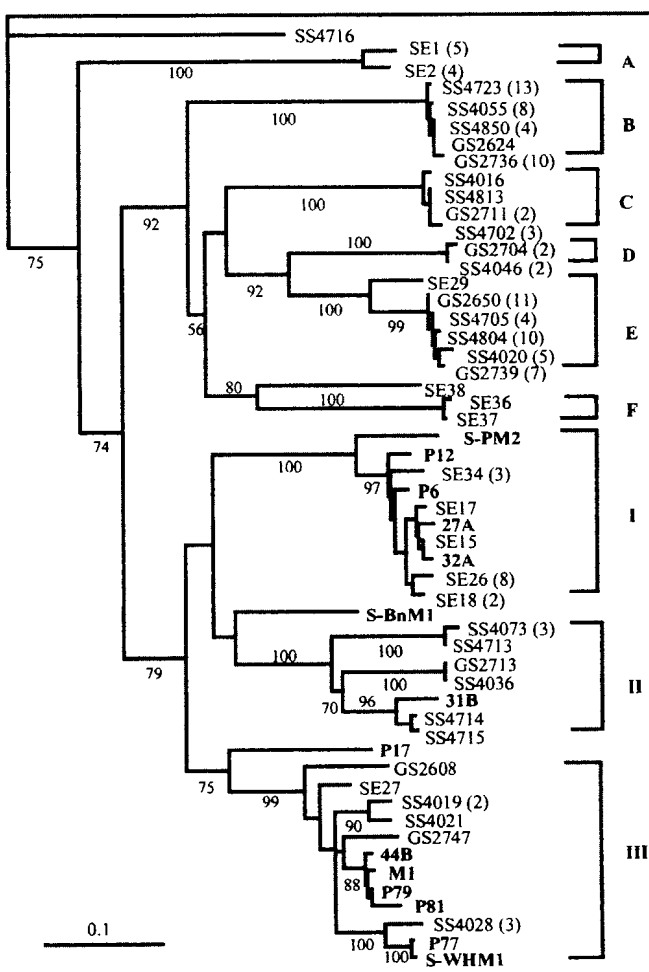
FIGURE 6.3 Neighbor-joining tree showing the phylogenetic affiliation of cyanophage iso-lates and representative clones from all six of the natural virus communities studied. The tree was constructed on the basis of a 176-amino-acid sequence alignment with T4 as the outgroup. Each value in parentheses is the number of different nucleotide sequences in the same cluster and same community as the representative clone. Clusters A through F and I through III were assigned on the basis of phylogenetic relatedness. Bootstrap values of less than 50 were not shown. The scale bar indicates 0.1 substitution per site.

bacteria of the natural consortium. However, some observations suggested that bacteriophages isolated from very low-nutrient marine habitats showed a trend toward increased breadth of host range. If confirmed, this could represent an adap-tation to the low host cell concentrations.

The still-unsettled subject of possible selection for broader host specificity in nutrient-poor marine environments is of substantial scientific relevance. If phages

with unusually broad target ranges are indeed more widely distributed in the marine environment than we expect from laboratory infections, transfer of genetic material between marine bacterial species via transduction might occur at even higher frequency than currently anticipated on the basis of the high concentrations of phages and bacterial cells and the tremendous volume of water in the oceans. Transduction, the accidental transfer of host DNA via a phage particle, occurs at varying rates for different phages, but is about once in every 10^8 phage infections in several well-studied systems. A mathematical treatment of experimental data from the estuary of the size of the Tampa Bay led to an estimation of 10^{14} transduction events occurring annually (Jiang and Paul, 1998). If even a minute fraction of this DNA is travelling between different bacterial species, it is clear that marine phages open up enormous possibilities for horizontal DNA transfer. The probability of *transformation* between unrelated species may well also be increased by the liberation of free bacterial DNA during lysis of infected cells. Current bacterial genomic analyses underline the important impact of lateral gene transfer, but despite the theoretical importance of transduction, few transduction studies have been conducted in the marine environment. Experiments with *Pseudomonas* revealed that higher transduction rates were obtained when both the donor and the recipient were lysogenic (Morrison et al., 1978). The reason is that a lysogenic strain can accept foreign DNA from a transducing phage, but is protected from lysis due to the immunity functions of the resident prophage if the two phages are related. In freshwater samples, suspended particles increased the transduction frequency since they allow adsorption of the donor and recipient cells on a solid phase.

6.4.   SOIL AND PLANT-ASSOCIATED PHAGES

The constant mixing found in aqueous contexts plays a major role in our ability to make generalizations about marine phages. Phages are also present at high levels in a variety of soils, but are much harder to study in detail and such work is in its infancy. Conditions in terrestrial environments vary far more drastically than they do in marine environments. One is really talking about enormous numbers of microenvironments, affected by position within soil particles, patterns of rain, drought, and temperature, and diurnal and seasonal variations, often with low and variable levels of exchange between them. Bacteria have many physiological adaptations for dealing with these changes, and these in turn affect host-phage interactions in ways that we are scarcely beginning to understand. In addition, phage have the challenge of finding a new bacterial host under conditions where the soil may often be only partially hydrated and the phage may be trapped in biofilms, bound to clay, or inactivated by acidity or other properties of the soil.

High phage concentrations comparable to those in marine environments have also been reported in terrestrial environments. For example, rhizosphere soil from a sugar beet field revealed 10^7 phage per gram by using transmission electron microscopy (Ashelford, 2003) and reconstitution experiments suggested that this figure underestimates the true number by nearly a factor of 10 for technical reasons. Phage from non-pathogenic bacteria like thermophilic *Bacillus* species are easily isolated from soil, compost, silage, and rotting straw, suggesting a tight association

of phage with plants (Sharp et al., 1986). A wide variety of phages were observed, most of them strain-specific within a given *Bacillus* species. Hybridization experiments with *Serratia* and *Pseudomonas* colonies from the soil showed that at least 5% of the bacteria are actually phage-infected. There are also reports of plants and plant extracts that can induce bacterial lysogens (Erskine, 1973; Gvozdyak, 1993; Sato, 1983). Soil phages, like their aquatic counterparts, are thus likely to be important in controlling bacterial populations and mediating gene transfer.

Various reports indicate that phage-host interactions can be quite complicated in the soil. For example, phages had only a minimal impact on net growth of Streptomycetes in the soil (Burroughs et al., 2000). In a combination of experimental observations and mathematical modeling, spatial heterogeneity in phage-host interaction, and temporal changes in susceptibility to phages were explored as determinants in bacterial escape from phage lysis in the soil. It turned out that germinating spores were more susceptible to phage infection than hyphae of developed mycelia. Mature resistant mycelia adsorb most of the *Streptomyces*-specific soil bacteria and thus protect younger susceptible hyphae from infection.

A number of labs have explored phages for biocontrol of plant pathogenic bacteria, as discussed in Chapter 13. One popular candidate is *Erwinia amylovora*, the cause of fire blight disease of apple and pear trees. Fire blight has generally been fought with limited success by antibiotics. Biological control by apathogenic *Pseudomonas* or by *Erwinia* phage Ea1 has been explored. *Erwinia*-specific phages like Ea1 were prevalent in orchards affected by fire blight, demonstrating a wide distribution of this phage; other genetically distinct phages, some with very broad host ranges on the fire blight pathogen, were also detected (Schnabel and Jones, 2001). The logistics of studying phage treatment of tree pathogens has been very challenging, but work is also going on in Guelph, Tbilisi, and elsewhere in applying phage against *Erwinia* species that infect important but more experimentally tractable model systems such as potatoes, carrots and ornamental flowers. In all cases, it has been possible to isolate a range of relevant phages from the wild, once again emphasizing their ubiquity in the environment. Phages against *Leuconostoc* and *Lactobacillus* have been isolated from numerous spontaneous fermentation processes, including coffee, pickled cucumbers, sauerkraut, cereals, and wine. These presumably represent phage that are normally associated with the respective plants being used for the fermentation processes.

6.5. ANIMAL-ASSOCIATED PHAGES

6.5.1. INTRODUCTION

When the draft sequence of the human genome arrived at the finishing line, it provided only a small part of the genetic material that makes up a human being. In fact, we harbour in our gut more bacterial cells than we have human cells. Not surprisingly, these gut bacteria are associated with their specific phage communities (Breitbart et al., 2003). This situation is not peculiar to humans; phage concentrations up to 10^9 per gram of feces were detected in cattle and sheep (Furuse, 1987). Other vertebrates (birds, fish) also contained appreciable phage concentrations in the gut

content. Phages have also been isolated from fecal pellets of many invertebrates belonging to diverse taxonomical groups (earthworms, bees, flies, mussels), as discussed by Ackermann and Dubow (1987). Oysters are filtering water and retain material suspended in the water, so it is no surprise that up to 10^6 pfu of vibriophages were found per g of oyster tissue (Baross et al., 1978b).

A number of studies have reported large phage populations in the rumens of sheep and cattle, affecting the complex balance among the bacteria that convert grass into nutrients to support ruminant growth. This is one area where research can be carried out at a level of sophistication comparable to that seen in the marine and the dairy environments. Animals with permanent rumen cannulae have facilitated non-invasive sampling and allowed studies over time that give new insight into rumen ecology—an important field in science-based livestock husbandry, which aims to optimize the efficiency of converting feed into meat and milk. Klieve and Bauchop (1988) partially purified phages from fluid samples collected through a nylon stocking from the rumens of cattle and sheep and studied them by electron microscopy. They found mainly tailed phages, with a high range of diversity as determined by head shape and size and tail morphologies: at least 14 different kinds of isometric-headed myoviridae, 4 podoviruses, and 4 isometric and 2 prolate-headed siphoviruses, including an astonishing giant phage with a head measuring 85×238 nm. The tails ranged from 25 to 1050 nm long. The ruminal phage DNA varied in size from 10 to 850 kbp. Klieve and Swain (1993) saw discrete bands of a wide variety of sizes, each essentially homogeneous, that differed in distribution from sample to sample, against a broad background of DNAs between 30 and 200 kbp; they showed that the latter represented a large, mixed population of intact DNAs, not degraded DNA from larger bands. The total phage population was determined to be about 10^{10} per ml—significantly higher than most earlier estimates. Klieve et al. (1989) also explored the incidence of mitomycin C-inducible temperate phage in the rumen. Of the 38 different ruminal bacteria that they analyzed, only 9 produced phage-like particles, all but one of them siphoviridae.

Phage can be isolated from the feces of most animals. In fact, an extended scientific discussion deals with the question of whether phages can be used as surrogate measures of fecal contamination levels in the environment. Since F^+-specific *E. coli* phages were mainly isolated from animal feces and *Bacteroides fragilis* phages only from human feces, specific phage detection methods can potentially differentiate the origin of environmental fecal contamination and phages were used as tracers to follow the intrusion of polluted surface waters into groundwater. The recent excitement about phage therapy of human and animal diseases has renewed the interest of microbiologists in the ecology of phage-bacterium interaction in the context of their hosts. Here, we will discuss three ecological niches: the gut, the oral cavity, and the skin.

6.5.2. ENTERIC PHAGES

The human gut is a complex ecosystem, colonized by about 400–500 microbial species, 30–40 of which account for 99% of the total population. A first understanding of human colonic phages comes from recent metagenomic analyses of an uncultured
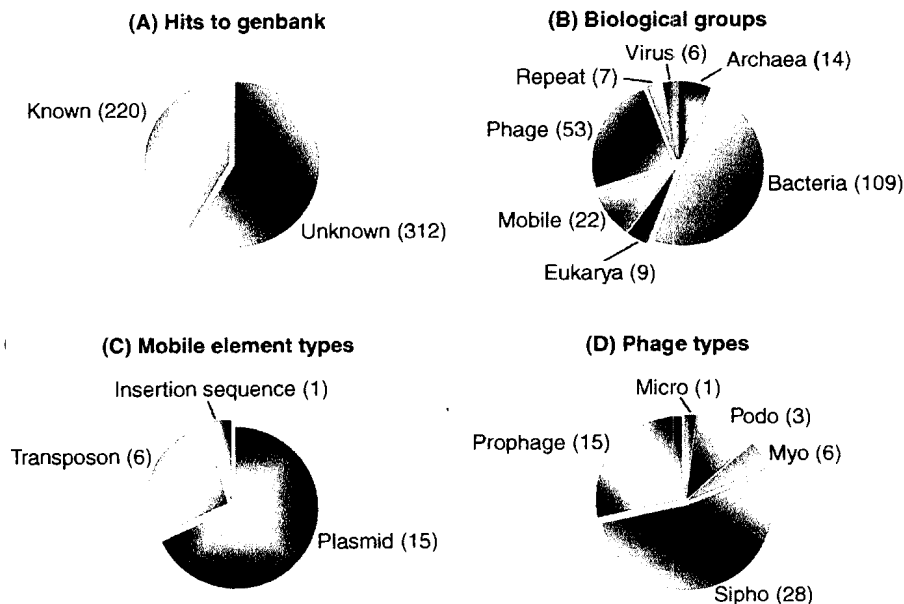
(A) Hits to genbank

(B) Biological groups

Virus (6) Archaea (14)
Repeat (7)
Phage (53)
Known (220)
Bacteria (109)
Mobile (22)
Unknown (312)
Eukarya (9)

(C) Mobile element types

Insertion sequence (1)
Transposon (6)
Plasmid (15)

(D) Phage types

Micro (1)
Podo (3)
Prophage (15)
Myo (6)
Sipho (28)

FIGURE 6.4 Genomic overview of uncultured viral community from human feces based on TBLASTX sequence similarities (Breitbart, 2003).

phage community from human feces of a single subject (Fig. 6.4) (Breitbart et al., 2003). DNA analysis using pulse field gel electrophoresis showed major bands at 15 and 90 kbp with minor bands at 30, 40, and 60 kbp and an average size of about 30 kbp—a significantly different distribution than that observed in seawater or the rumen, as described above; the dominant band at only 15 kbp was especially unusual. Extracted DNA was cloned and sequenced. No significant GenBank hits were seen for 59% of the 532 sequences. Half of the positive hits were related to bacterial genes, while a quarter were with phage genes, mainly to structural proteins and terminases; one might expect that a large fraction of the sequences without GenBank hits were from phages, since more information is available from bacterial genome projects and sequenced phages often show a very large fraction of unique sequences. There were few matches to T7-like podoviridae or to λ-like siphoviridae, which were the most abundant species observed in the marine environment. Many were to phages infecting gram-positive bacteria, in agreement with the observation that 62% of the cells detected in human feces with specific rRNA probes were gram-positive bacteria. Since earlier studies exploring gut phages against *E. coli*, *Salmonella* and *Bacteroides* have shown substantial differences among individuals that did not correlate with age or sex, it will be very important to carry out more studies of this nature and also look for correlations with such factors as dietary patterns and prior antibiotic use.

    E. coli and its phages belong for molecular biologists to the most carefully investigated phage systems, yet surprisingly few reports have investigated the gut

ecology of coliphages or even the ability of coliphages to replicate under *anaerobic* conditions. Kutter et. al. (1994) reported that phage T4 can successfully infect anaerobically as long as the bacteria are already *pre-adapted* to anaerobic growth. In this connection, it is important to distinguish *anaerobic fermentation*, in which bacteria use an organic byproduct of metabolism as the terminal electron acceptor, from *anaerobic respiration*, in which they use an electron acceptor such as nitrate or fumarate. The former is found, for example, in the rumen of grass-eating mammals, where much glucose is available, while the latter predominates in the colon, where virtually no fermentable substrates remain. For T4-like phages, we find quite different patterns of infection in comparing anaerobic respiration, anaerobic fermentation and aerobic respiration, even when largely similar defined media are used; burst sizes are generally lower anaerobically and lysis is often delayed for many hours, even though cells lysed with chloroform at 50 minutes after infection show good phage production.

In one large study, stool samples from 600 healthy patients and 140 patients suffering from traveller's diarrhea were investigated for the presence of coliphages on 10 different *E. coli* indicator strains (Furuse, 1987). From healthy subjects, 34% of the stool samples contained phages but only 1% showed high amounts. Most of them were classified as temperate phages related to phages phi80, lambda, and phi28. In comparison, 70% of the stools from diarrhea patients (half of whom might have had an enterotoxigenic *E. coli* infection) contained phages, 18% in high concentrations. About half of these isolates were composed of virulent phages related to T4 and T5. This change of phage composition between healthy subjects and diarrhea patients seemed to reflect some disturbance of their intestinal bacterial flora. In another study with stool samples from 160 pediatric diarrhea patients from a clinic in Dhaka Bangladesh, a third of the patients were phage-positive when tested on two *E. coli* indicator cells (Chibani-Chennoufi et al., in preparation). Notably, in this clinic about 30% of cases are caused by *E. coli* (Albert et al., 1995). The phages were nearly exclusively T4-like phages by genome size and morphology. However, restriction analysis, diagnostic PCR and partial sequencing demonstrated substantial genetic diversity between the phage isolates. The stool samples from the Bangladeshi children in the convalescent phase yielded no higher phage counts than the stool samples from the same patients during the acute diarrhea episode, but the phages were not screened on the infecting *E. coli* serotypes. Strain-specific phages might thus suffer from underreporting. In animal experiments (Chibani-Chennoufi et al., 2004) and recent human adult volunteer trials at the Nestle Research Centre (Bruttin et al., manuscript in preparation), T4 and T4-like phages were added to the drinking water. The phages survived the gastric passage and were recovered from the feces in titres comparable to the dose orally fed to the subjects.

This suggests that these phages transit through the entire gut relatively unscathed, but no significant replication on the endogenous *E. coli* gut flora was observed despite the fact that many fecal strains were susceptible to the phage infection *in vitro*; fecal counts of the endogenous *E. coli* flora also remained relatively unchanged both in mouse and in man. These experiments raise doubts about too-simple models of phage-host interaction in the mammalian gut. The metabolically active *E. coli* dwelling as microcolonies in the intestinal mucus layer covering the mucosa might be physically

protected against lumenal phage (Poulsen, et al., 1995; Krogfelt, et al. 1993). Mouse experiments suggested that freshly added *E. coli* applied by mouth were susceptible to luminal phage. Actually, we know relatively little about the ecology of *E. coli* in the human gut. *E. coli* might even be a misnomer since the pathogenic strains cause their diarrhea effects by interacting with the small intestine and not the colon. Mucosa-associated *E. coli* in the small intestine might acquire sufficient oxygen from the blood vessels to facilitate host and phage growth. Here again, research areas at the borderline between microbiology, ecology and physiology are key. It is clearly very important to develop a better general understanding of phage infection under such conditions and of the roles of phage in gut and rumen ecology. These ecological considerations are important for phage therapy approaches in the alimentary tract. (The Hungate technique, an inexpensive and fairly simple system for carrying out anaerobic phage infection studies in vitro, is described in the Appendix.)

 E. coli and its phages can be easily isolated from the environment. For RNA coliphages, the most common sources were sewage, both from domestic drainage and sewage treatment plants, followed by feces of man, domesticated animals (cows, pigs), and zoo animals. Much less rich sources were environmental water samples (Furuse, 1987). This led to the proposal that coliphages could be a surrogate measure for fecal contamination of recreational waters or other waters of public health interest (el-Abagy et al., 1988). Recently it has become technically possible to screen for human viruses in water samples. However, the majority of the medically important human enteroviruses are RNA viruses, with the single exception of adenovirus. The most sensitive PCR techniques thus cannot be applied. Testing in coastal waters in California impacted by urban run-off water revealed that four out of twelve sites contained adenovirus. However, coliform bacteria and coliphages did not correlate with the adenovirus, calling for a reevaluation of both indicator organisms for the monitoring of recreational waters. In contrast F-specific RNA coliphages showed a good correlation with adenoviruses (Jiang et al., 2001). There is some ecological knowledge on these RNA coliphages in the environment (Furuse, 1987). They are found with strikingly variable prevalence in domestic drainage from different geographical areas. These phages were also found in the feces of humans and domesticated animals. The fact that the feces from cows and pigs contained large amounts of RNA coliphages suggested that these phages were actually propagated in the intestines of the animals. Different groups of RNA coliphages were found with distinct frequencies in humans and animals, suggesting some specificity that probably reflected the distinct composition of the gastrointestinal microbial flora. This fact supports the idea that the intestine of mammals may constitute one of the natural habitats of coliphages despite the fact that *E. coli* represents only a minor constituent of the normal bacterial flora in the human alimentary tract

6.5.3. OTHER PHAGE SITES OF INTEREST: ORAL CAVITY AND VAGINA

Bacteriophages have also been isolated from other parts of the alimentary tract, for example the oral cavity. This anatomic site is richly colonized with many mainly commensal bacteria, but pathogenic strains like *Streptococcus pneumoniae* and

S. pyogenes were also detected. The actual species composition varies from precise anatomic site to site. Bacteriophages play an important role for these pathogens, since a majority of the clinical isolates of *S. pneumoniae* are lysogenic (Severina et al., 1999; Ramirez et al., 1999) and in the case of *S. pyogenes* the prophages contribute a set of virulence factors to the cell that directly influence the epidemiology of the clinical isolates (Beres et al., 2002). Phage lysins applied to the oral cavity can potentially diminish the degree of colonization of the oral cavity with these pathogenic bacteria and the seeding of these pathogens into the respiratory tract (see Chapter 12). In about 3% of dental patients, dental plaque yielded both *Actinomyces* and the corresponding phages (Tylenda et al., 1985). The phages could be re-isolated from most of the patients up to a month later, suggesting that they belonged to the local microbial community. In another study, about 10% of the oral washings from dental patients allowed the isolation of virulent phages directed against *Veillonella* strains, a resident constituent of the oral cavity. *Enterococcus faecalis* phages were isolated from human saliva (Bachrach et al., 2003), but the ecological role of all these oral phages is still unsettled.

Recent data suggest that phages may play an important role in the ecology of the vagina as well, in a way that is attracting significant medical attention (Kilic et al., 2001). Lactobacilli constitute the dominant vaginal bacterial flora and are beneficial to women's health, since they inhibit the growth of harmful microorganisms by producing lactic acid, hydrogen peroxide and other antimicrobial substances (Redondo-Lopez et al., 1990). Bacterial vaginosis, linked to various medical conditions, is observed when anaerobic bacteria outnumber lactobacilli in the vagina. About 30% of lactobacilli isolated from healthy women from the United States or Turkey were lysogenic. This rate was 50% in women with bacterial vaginosis. Many of these lysogens could be induced by mitomycin C, releasing infectious phage, some at high titer, that could lytically infect lactobacilli belonging to multiple species (*L. crispatus, jensenii, gasseri, fermentum,* and *vaginalis*). The authors note further that smoking is a risk factor for bacterial vaginosis, and the mutagen benzopyrene, which is created by smoking tobacco, could induce phages from lysogenic lactobacilli at the concentrations found in vaginal secretions of smoking women (Pavlova and Tao, 2000). They suggest that smoking may reduce vaginal lactobacilli by promoting phage induction, leading to a replacement of lactobacilli by anaerobic bacteria and precipitating bacterial vaginosis.

6.6. INDUSTRIAL PHAGE ECOLOGY

6.6.1. DAIRY INDUSTRY

Phage contamination is a constant problem for fermentation-dependent industries such as the dairy industry. In fact, phages are the primary cause for fermentation delays in yogurt and cheese production and can in extreme cases lead to the loss of the product. These cost considerations were a powerful incentive for the dairy industry to design elaborate phage control measures. An overview of this activity is provided in the flow scheme of Fig. 6.5. The first steps in these control measures are ecological surveys of phages in the factory environment. The industry needs to
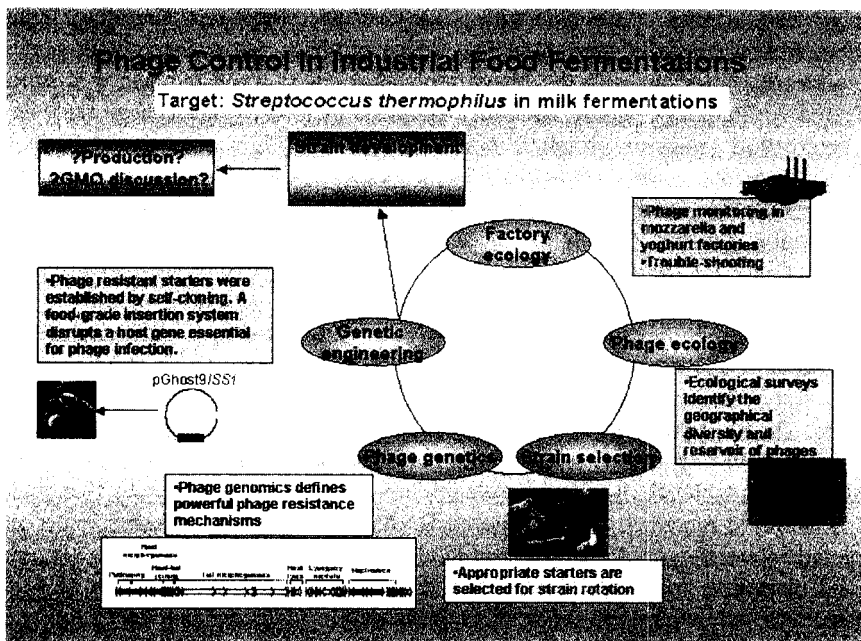
FIGURE 6.5 Phage control in industrial food fermentation. The flow scheme illustrates the approach in the company of one of the authors using *Streptococcus thermophilus* as an example. The different steps depicted in this flow diagram have been the subject of a number of publications. A recent review summarizing this work can be found in Brüssow (2001).

know the extent of the problem, the prevalence and titers of the phages, and their distribution in space and time. The dairy industry has accumulated substantial knowledge in the field of phage factory ecology, but these data are generally not published as the ecology of phages in a man-made environment has not attracted many academic ecology-oriented microbiologists. However, industrial microbiologists soon realized that it is not sufficient to explore the distribution of phages in the factory. The space around the factory and the material delivered into the factory (milk and starters) had to be studied as well. Classically, the major focus of this industrially-oriented work is the selection of appropriate combinations of bacterial starters showing non-overlapping phage susceptibility patterns to allow the design of an efficient starter strain rotation system, which is still the most-used means of coping with the industrial phage problem.

Over the last decade, substantial efforts have been conducted to design phage-resistant starter strains (see also Chapter 10). In the case of cheese production using *Lactococcus lactis* as a starter, this task was facilitated by the availability of many natural phage resistance systems in this bacterium. This allowed the construction of phage-resistant starters by the transfer of plasmids using methods that are not considered genetic engineering. The situation is different for *Streptococcus thermophilus*, the bacterial starter used in yogurt production. Few strains contain plasmids

and even fewer possess plasmids with phage-resistance functions. Therefore, industrial microbiologists did substantial sequencing of phage genomes to obtain genetic elements that interfere with the phage replication cycle when the starter is super-infected with a phage. These inhibitory elements included genes (phage repressor, superinfection exclusion proteins), non-coding DNA (origin of phage replication) and anti-messengers of phage genes. These systems suffer from the fact that they are metabolically costly to the cell; they are most efficient when present on high copy number plasmids, and frequently do not work when integrated into the bacterial chromosome as a single copy. In addition, food-grade plasmid vectors have only been developed for some bacterial starters (*L. lactis*).

In *S. thermophilus*, this problem could be circumvented by genetic engineering approaches where a plasmid is forced into chromosomal integration, leading to the disruption of bacterial genes. Phage-resistant mutants are then selected and characterized. The most powerful resistance mechanism was the disruption of a membrane protein that was apparently used by the phage for the injection of its DNA into the cell (see Chapter 7). Serial passaging led to loss of the plasmid, while the IS element of the plasmid remained in the bacterial DNA and disrupted the expression of the targeted bacterial gene. As the plasmid IS element occurs naturally in dairy bacteria, phage resistance can thus be achieved by self-cloning. However, none of these approaches in *S. thermophilus* were introduced into industrial practice. European, in contrast to US, legislation requires the labelling of starters as GMO (genetically modified organisms) that were modified by self-cloning and thus contain only species-specific DNA. The persistent scepticism of the European consumer towards GMO in food production led to a substantial reduction of research activity in the dairy sector, both in industry and in EU-funded public research. Nevertheless, the search for practical solutions to the industrial phage problems made dairy phages one of the best-investigated phage systems with respect to both phage genomics analysis and phage ecology. Pertinent ecological features are analysed in the following paragraphs. As this research was conducted in the private sector, only part of the data has been published.

Two basic kinds of ecological situations can be distinguished in the industrial food-fermentation environment, as characterized by the yogurt factory and the cheese factory. Even if the same bacterial starter is used (yogurt and mozzarella cheese fermented by added *S. thermophilus*), the two dairy factories differ in several basic respects. Milk for yogurt production undergoes treatment at 90°C, which kills all phages (Quiberoni et al., 1999), while raw or pasteurised milk is used in cheese fermentation. Furthermore, yogurt production is a relatively aseptic process where the fermented product has minimal exposure to the factory environment. In contrast, during cheese making the factory experiences a massive daily aerosol contamination during cheese whey separation (Budde-Niekiel et al., 1985). In Europe, industrial yogurt factories are generally smaller than cheese factories (about 50,000 vs. 500,000 liters of milk processed daily per factory). Phages are thus seldom seen in yogurt production, though they may unknowingly be introduced into the factory either by the starter cells or by interventions that compromise the physical barrier separating the product from the environment. Phage problems are still sufficiently frequent to make them the primary source of fermentation failure in yogurt

production—mostly in the form of fermentation delays or product alterations, but occasionally also as complete product loss. Cheese factories, in contrast, are characterized by the coexistence of phage in the milk and bacterial starter and thus this is a constant problem.

Yogurt samples in a factory reporting occasional fermentation delays yielded phage-positive samples with titres up to 800 pfu/ml. Aerosols containing phage were the likely vehicle for phage transmission within the factory. In the literature, two potential sources of phage were identified: raw milk (Bruttin et al., 1997) and lysogenic starter strains (Heap et al., 1978). These strains spontaneously release phage that can lead to phage amplification if susceptible starter cells are used in the same factory. Fermentation becomes prolonged when the phage titres mount beyond a critical threshold of 1,000 or 10,000 pfu/ml. When a rotation system is used, regular fluctuations of the phage titres synchronized with the starter strain rotation can be observed. This situation can be maintained over some time. When the titre of the phage rises beyond 10^6 pfu/ml, a fermentation failure is the likely consequence and cannot be buffered by a rotation system. The milk samples inoculated with the starter will no longer coagulate and the product is altered in its technological properties or entirely lost. The production line must then be carefully cleaned to eliminate the phages. If available, starters insensitive to the phage of the fermentation failure are sometimes used to prevent a recontamination of the production line by residual phage in the factory.

Persistent phage infection is frequently observed in cheese factories. In mozzarella fermentation using a complex mixture of *S. thermophilus* starter strains, phage titres in the cheese whey were normally 10^5 pfu/ml or higher (peak titres: 10^7 pfu/ ml) (Bruttin et al., 1997). All cheese whey samples contained between four and eight different phage strains. Some phage types were frequently observed and showed high titres while others were only occasionally seen, at low titres. No apparent regularities could be deduced from the temporal cycling of the specific phage titres in the whey samples. In fermentation simulations, cell counts dropped when phage were added at an moi (multiplicity of infection) of 0.01 and cells were lost and no coagulation occurred when the cells were infected at a moi of 0.1. At low moi, two successive waves of phage replication were observed. High yields of progeny phage were only obtained with cells in active growth. Phage yields dropped by 5 orders of magnitude when cells were infected in the late logarithmic or stationary phase. However, when these cells were resuspended in fresh medium and growth resumed, renewed phage replication was observed, leading to lysis and phage release. In contrast, infected cells maintained in the stationary phase failed to produce sizable amounts of progeny phage.

No phage-resistant cells are selected in the factory ecology, since the fermentation process is restarted each time with a frozen standard starter culture. A repetition of the classical Delbrüeck-Luria phage challenge experiment (Luria and Delbrück, 1943) yielded only very few outgrowing cells that had lost the capacity to adsorb the challenge phage. These mutant cells grew poorly, suggesting a metabolic cost for mutation to phage receptor loss in *S. thermophilus* (unpublished results).

S. thermophilus is found in raw milk after enrichment techniques (heating at 60°C) and has never been isolated from any sources not in contact with milk.

In contrast, its closest relative, *S. salivarius*, is an oral commensal. However, total bacterial counts in uncontaminated raw milk are relatively low: about 1000 cfu/ml. It is thus not surprising that *S. thermophilus* phages can only be isolated from raw milk in low titers, ranging from undetectable (<10/ml) in most samples to a maximum of 130 pfu/ml (Bruttin et al., 1997). This poses a dilemma: How can phages be maintained in the environment when they have only such a small pool of susceptible cells? The most logical explanations might be lysogeny and wide host range. However, the analysis of hundreds of *S. thermophilus* phage isolates from cheese factories showed very narrow host ranges. All but two phages were only able to infect the strain on which they were isolated (Bruttin et al., 1997). This pattern of strain-specificity was also observed in larger industrial strain collections (Le Marrec et al., 1997). Furthermore, lysogenic strains are rare in industrial strain collections; only about 1% of strains can be induced by mitomycin C, for example. Southern hybridization with DNA of the two major classes of *S. thermophilus* phages confirmed a low lysogeny rate. Only a single survey reported a 10% rate of lysogenic cells by hybridization (Fayard et al., 1993). In addition, less than one per cent of *S. thermophilus* phages from major strain collections are temperate phages (Brüssow et al. 1994; Le Marrec et al., 1997; Lucchini et al., 1999b). However, the genome maps of the major virulent *S. thermophilus* phages betray their origin from temperate parental phages (Lucchini et al., 1999a; Bruttin and Brüssow, 1996). The preponderance of virulent phages in our collections might therefore represent an adaptation to the abundance of host cells in the dairy environment. In fact, serial passage of a temperate *S. thermophilus* phage quickly resulted even in the laboratory in its replacement by a virulent derivative deletion mutant. The rare raw milk *S. thermophilus* phages are nevertheless the source of phage contaminations in the cheese factory. In a large intervention trial, one starter combination was replaced by a second that was insensitive to the resident phages of the factory. The intervention resulted in a nearly immediate disappearance of the resident phages: 70% of the milk samples lacked any phages and 30% contained phages detectable only on the old starters (apparently a washout from the previous high phage contamination level since the titres were low). However, by 5–7 days after the intervention the first three phages infecting the new starters were detected. Restriction analysis of the phage DNA traced the origin of the new phages to the rare raw milk samples delivered to the factory during the intervention period.

Cheese factories using *Lactococcus lactis* as a starter frequently yielded high levels of phages; one study reported up to 10^9 pfu/ml whey without great fluctuation. Phages were ubiquitous in the factory, and up to 10^5 pfu/m^3 phages were detected in the factory air (Neve et al., 1994). Like some streptococcal phages, lactococcal phages were reported to survive pasteurisation. *L. lactis* strains were frequently detected in the raw milk while lactococcal phages were only a rare observation. Many lactococcal phages showed very restricted host ranges, limited to one or a few starter strains within the *L. lactis* species. A clear difference from *S. thermophilus* phages is the much greater morphological and genetic variability of lactococcal phages; in some (unpublished) surveys, shifts in the predominant morphological types of phages were observed in the whey samples that correlated with the change of the starter culture.

6.6.2. NON-DAIRY FOOD FERMENTATION: SAUERKRAUT AND SAUSAGE

Like most vegetable fermentations, sauerkraut fermentation is spontaneous and relies on bacterial epiphytes present on cabbage. Food ecological studies demonstrated a succession of two groups of lactic acid bacteria. In the initial heterofermentative stage, *Leuconostoc* species dominate the fermentation. When the pH decreases, they are followed by the more acid-tolerant *Lactobacillus* species; *L. plantarum* eventually becomes the most abundant species. An ecological survey also demonstrated a succession of two phage populations corresponding to the replacement of *Leuconostoc* by *Lactobacilli* (Yoon et al., 2002; Barrangou et al., 2002; Marchesini et al., 1992; Lu et al., 2003b). The *Leuconostoc* phages represented a number of distinct *Sipho-* and *Myoviridae*, while the *Lactobacillus* phages included in addition *Podoviridae*. The *Myoviridae* showed genome sizes in the 40–50-kbp range. The *Leuconostoc* phages showed very narrow host ranges, infecting only selected strains of *L. fallax* species. In contrast one *Lactobacillus* phage could infect both of the ecologically-related species *L. brevis* and *L. plantarum*. *Leuconostoc* and *Lactobacillus* phages have been isolated from numerous other spontaneous fermentation processes: coffee, pickled cucumbers, cereals, and wine (Lu et al., 2003a; Lu and Dahlquist, 1992). Apparently, phage infections are not limited to food fermentation initiated with bacterial starter cultures.

Lactobacillus plantarum is also used as an industrial starter in meat fermentation. Phage infections were documented in salami production, but they were of no industrial impact. After an initial rise, the phage titres dropped and the initial phage-sensitive starter population was replaced by a phage-insensitive mutant derivative strain. From meat fermentation, a *L. plantarum* myovirus with large genome size was isolated (Chibani-Chennoufi et al., 2004). The 130-kbp genome showed close sequence similarity with *Bacillus* phage SPO1 at the protein level in regions encoding structural and DNA replication proteins. Both have been recently shown to be related to phages infecting other gram-positive bacteria such as *Staphylococcus* and *Listeria*, defining a broad new genus of *Myoviridae*, as discussed in Chapter 5. Phage infections were without consequence in salami fermentation using *Staphylococcus carnosus* starters that do not quickly develop phage resistance. Two reasons were quoted for the limited impact of phages on meat fermentation: The staphylococcal starter showed only a modest growth and the solid food matrix prevented the spread of phage in the food product (Marchesini et al., 1992).

6.7. OUTLOOK

All in all, recent ecological surveys in a variety of environments have underlined the notion that we live in a sea of bacteriophages, as they are common biological parts of the soil we stand on, air we breathe, water we drink, and food we eat. There is also an increased appreciation that phages play a key role in controlling bacterial population levels everywhere in the environment, and in the genetic diversification of bacterial strains and species. These observations clearly have profound basic- and applied-science implications, leading to important theoretical insights and concepts

for ecology and medicine. With the addition of such new techniques as metagenomic analyses, it is clear that this field will explode in the next few years, providing many new insights.

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