**Approaches to Studying Lysogeny**

**Introduction**

A bacteriophage (or phage) is a virus which infects bacteria ([Clokie et al., 2011](https://doi.org/10.4161/bact.1.1.14942)). If considered to be alive, phages would comprise one of the most abundant ‘organisms’ in the biosphere. All phages have the ability to act as predators of bacteria; however, temperate phages are capable of making the choice of predating upon bacteria and living in a symbiotic relationship with their host. Phage population dynamics driven by this choice may affect bacterial population dynamics in an ecosystem, which may have significant effects on the ecosystem as a whole.

**Terminology**

This section aims at explaining some of the most commonly used terms in phage literature, as some of the terminology may be confusing to new readers.

**Explanation of Terms in Phage Literature**

A **virion** is a complete viral particle consisting of a nucleic acid genome encapsulated by a protein capsid, which is capable of infecting a new cell once released from its old host ([Hobbs & Abedon, 2016](https://doi.org/10.1093/femsle/fnw047)). A virion which has been released from a host and which therefore resides extracellularly is called a **free phage.** Once a free phage binds to a host it is capable of infecting, it releases its genetic material inside of the host. A successful infection usually results in the initiation of a process capable of replicating the phage genome, which may include one of the following reproductive strategies.

A **productive cycle** is a term for a phage infection resulting in the production of virions ([Hobbs & Abedon, 2016](https://doi.org/10.1093/femsle/fnw047)). The most common productive cycle is the **lytic cycle**, which produces many virions within a short period of time and releases them by lysing the host. Another, less common productive cycle is the **chronic cycle**, which results in a continuous production and release of virions from the host cell, usually without killing the host. In contrast, the **lysogenic cycle** does not result in the immediate production of virions. Instead, the phage integrates itself into the host chromosome or extrachromosomal elements, becoming a **prophage**. A bacterium which is infected by a phage undergoing the lysogenic cycle, therefore having a prophage incorporated into its genome, is called a **lysogen. Lysogeny** is a term for an infection caused by the lysogenic cycle. A relevant historical definition defines lysogeny as the “hereditary power of [an infected bacterium] to produce bacteriophage” ([Lwoff, 1953](https://www.academia.edu/33489587/Disambiguating_Bacteriophage_Pseudolysogeny_An_Historical_Analysis_of_Lysogeny_Pseudolysogeny_and_the_Phage_Carrier_State)). This “hereditary power” refers to the fact that the descendent of a lysogen will inherit its ancestor’s prophage, therefore resulting in vertical transmission of the phage. The ability to “produce bacteriophage” refers to the ability of a prophage, under certain conditions, to undergo **induction**, initiating a productive cycle to produce virions. Occasionally, a phage may undergo **pseudolysogeny** and will not initiate either a productive cycle or a lysogenic cycle upon infection ([Łoś & Węgrzyn, 2012](https://doi.org/10.1016/b978-0-12-394621-8.00019-4)). This may be due to the phage infecting a starving bacterium, which lacks the resources to produce phage components.

Phages may be classified according to their ability to enter a productive or lysogenic cycle. A **temperate phage** is capable of entering the lysogenic cycle as well as a productive cycle ([Hobbs & Abedon, 2016](https://doi.org/10.1093/femsle/fnw047)). However, a temperate phage integrated into a host genome may lose its ability to undergo a productive cycle, therefore becoming a **cryptic prophage (or defective phage)** ([Campbell, 1998](https://doi.org/10.1007/978-1-4615-6369-3_3))**.** In contrast, an **intemperate phage (or non-temperate phage)** is incapable of entering the lysogenic cycle  ([Hobbs & Abedon, 2016](https://doi.org/10.1093/femsle/fnw047)). A **virulent phage** **(or obligately lytic phage or strictly lytic phage)** is an intemperate phage which may only replicate using the lytic cycle. Such a phage may or may not be recently descended from a temperate phage which has lost its ability to enter the lysogenic cycle. This differs from a **professionally lytic phage,** which is also an intemperate phage which may only replicate using the lytic cycle, but is not recently descended from a temperate phage.

**Possible Points of Confusion**

There exist some potential points of confusion within the terminology of phages, many from alternative historical definitions of common terms. Historically, the term **virulent phage** has also meant a phage which was adept at destroying bacterial populations ([Hobbs & Abedon, 2016](https://doi.org/10.1093/femsle/fnw047)). Due to possible confusion regarding the term **virulent phage**, the term **obligately lytic phage** will be used instead where appropriate. Similarly, the term **lysogen** has its roots in the phrase “lysis-generating”, and was historically used to describe a population of bacteria capable of releasing factors (now known to be phages) capable of lysing a population it was transferred to. A **pseudolysogenic** **(or carrier)** strain of bacteria was historically used to describe a population of obligately lytic phage within a population of mostly resistant bacteria, where a minority of sensitive bacteria allowed continued propagation of a phage ([Adams, 2009](https://www.academia.edu/33489587/Disambiguating_Bacteriophage_Pseudolysogeny_An_Historical_Analysis_of_Lysogeny_Pseudolysogeny_and_the_Phage_Carrier_State)). However, not all confusion is due to historical definitions. For example, the term **virulent phage** has sometimes used to mean an intemperate phage, regardless of whether or not it has been descended from a temperate phage ([Hobbs & Abedon, 2016](https://doi.org/10.1093/femsle/fnw047)). Additionally, in the growing field of phage therapy, a **lytic phage** is a term for an obligately lytic phage, while a **lysogenic phage** is a term for a temperate phage.

**Glossary of Common Terms Used In This Article**

* **Free phage** - An extracellular virion.
* **Induction** - An event during which a phage undergoing the lysogenic cycle initiates a lytic cycle.\*
* **Lysogen** - A bacterium infected with a phage undergoing the lysogenic cycle.
* **Lysogenic cycle** - A phage reproduction strategy resulting in the integration of the phage genome into the bacterial genome, resulting in the reproduction of the phage genome when the host reproduces.
* **Lysogeny** - Infections caused by a phage undergoing the lysogenic cycle. Lysogeny-related studies may either directly study some aspect of these infections, or may observe a factor thought to correlate with the increased frequency of lysogeny in an ecosystem.
* **Lytic cycle** - A phage reproduction strategy resulting in the production and release of many virions within a short period of time, resulting in the lysis of the host.
* **Metagenomics** - A discipline involving the collection and analysis of genetic material from all organisms in a sample.
* **Obligately lytic phage** - A phage which may only replicate using the lytic cycle. May or may not be descended by a temperate phage. Used instead of the more common term ‘virulent phage’, as the term ‘virulent phage’ has multiple definitions.
* **Phage** - A virus which infects bacteria. Synonym for ‘bacteriophage’.
* **Prophage** - A phage genome integrated into a bacterial genome.
* **Temperate phage** - A phage which is capable of entering the lysogenic cycle.
* **Virion** -  A completed viral particle consisting of a nucleic acid genome and a protein capsid, which may then be released extracellularly.

\* For the purposes of this article. For more information, see Explanation of Terms in Phage Literature.

**Studying Lysogeny**

Many different approaches from various fields of biology, such as microbiology, genomics, and molecular biology, have been used to study lysogeny. Some of these approaches, such as the intracellular visualization of phage, directly study lysogeny (the *infections* caused by the lysogenic cycle). Other approaches, such as the metagenomic studies measuring markers of temperate phage, indirectly study lysogeny by observing factors which are thought to correlate with the increased frequency of lysogeny in an ecosystem. This section provides a high-level conceptual overview of some of the methods which have been used, or which have proposed to be used, to study lysogeny. However, this does not constitute a comprehensive list of methods or studies in the field. **The main measure of lysogeny detected by each approach is bolded.**

**Forced Induction Studies**

*Additional Definitions:*

* *Average burst size:* The average number of virions produced per cell upon lytic cycle induction. Typically either assumed or measured by enumerating virions within bacteria using transmission electron microscopy ([Jiang & Paul, 1996](https://doi.org/10.3354/meps142027)).
* *Virus-like particles (VLPs)*: Particles of similar sizes to viruses, but which cannot be confirmed to be viruses through identification of viral attributes ([Jiang & Paul, 1996](https://doi.org/10.3354/meps142027)).
* *Direct counting:* A method of determining number of particles in a sample involving the division of the sample into several grids, followed by the enumeration of each particle in several randomly sampled grids and the extrapolation of this number to the rest of the sample ([Kirchman et al., 1982](https://aem.asm.org/content/aem/44/2/376.full.pdf)).

Many prophages have a low spontaneous induction rate, and thus may remain in their hosts for long periods of time ([Gandon, 2016](https://doi.org/10.1016/j.tim.2016.02.008)). However, prophages will induce a productive cycle (usually assumed to be lytic) when exposed to external stressors such as DNA damage. Based on this principle, an old, but widely accepted test for studying lysogeny in a system involves inducing productive cycles in a bacterial sample through administering induction agents such as ultraviolet radiation or mitomycin C ([Otsuji et al., 1959](https://doi.org/10.1038/1841079b0)). It would be expected that if lysogens exist in such a system, application of the induction-causing agent would lead to bacterial death and the production of virions. **Studies using this method typically estimate the proportion of bacteria in a system which are lysogens.**

To measure the proportion of lysogens in a system, the number of bacteria and the number of virions or virus-like particles (VLPs) are measured before and after an induction-causing agent is added to a bacterial sample. One common way to ascertain bacterial and viral counts is by direct counting using epifluorescence microscopy, which utilizes a fluorescent stain to stain the DNA of viruses and bacteria ([Noble & Fuhrman, 1998](https://doi.org/10.3354/ame014113)). Then, the proportion of lysogens may be estimated as the fraction of bacteria which died due to viral induction by the induction agent, adjusted for bacterial deaths due to the agent itself  ([Jiang & Paul, 1996](https://doi.org/10.3354/meps142027)). Alternatively, the number of lysogens may be estimated by dividing the increase in virions or VLPs by an estimated average burst size. One study which used these prophage induction methods in marine environments found that 54% of sampled samples showed significant prophage induction, indicating the presence of susceptible lysogens in these samples ([Jiang & Paul, 1996](https://doi.org/10.3354/meps142027)).

However, some problems exist with the technique of prophage induction. Fundamentally, the sensitivity to induction varies by bacterial and viral species ([Howard-Varona et al., 2017](https://doi.org/10.1038/ismej.2017.16)). For simplicity, it is usually assumed that all existing lysogens in a sample are induced, but this is likely an incorrect assumption. An accurate estimate of the proportion of lysogens using average burst size depends heavily on accurate burst size estimates, which requires additional steps to ascertain ([Parada et al., 2006](https://doi.org/10.1017/s002531540601352x)). Additionally, another issue stems from the issue of the identity of VLPs; one study has shown that the common epifluorescence microscopy used for viral enumeration may generate ‘fake virus particles’, overestimating the total number of phages present in the sample ([Forterre et al., 2013](https://doi.org/10.1016/j.tim.2012.10.005)).

**The Virus to Microbe Ratio**

*Additional Definitions:*

* *Transduction:* Transfer of genes between bacteria due to temperate phage; caused when prophages erroneously excise some host DNA during induction ([Chiang et al., 2019](https://doi.org/10.1371/journal.ppat.1007878)).

**The virus to microbe ratio, (VMR) measures the number of free phages per microbe** ([Cael et al., 2018](https://doi.org/10.3389/fmicb.2018.00358)). This term is commonly used interchangeably with the virus to bacteria ratio, as it is generally thought that the VMR is dominated by the interaction between bacteria and phage. Lower VMRs are indicative of conditions which are thought to evolutionarily favor lysogeny. From the perspective of phages, these environments may reduce the concentration of free phages due to high rates of extracellular virion decay or slow viral production rates ([Howard-Varona et al., 2017](https://doi.org/10.1038/ismej.2017.16)). From the bacterial perspective, these extreme environments likely pose a greater challenge to survival; thus, these bacterial populations may benefit from the additional genetic diversity conferred by transduction of genes by phages or from other phage-encoded genes improving fitness. Measurement of the VMR may be accomplished through directly counting bacteria and virions, using methods similar to those used in forced induction studies. Then, the virion count may be divided by the bacterial count to find the VMR. One issue with this measurement is that it does not appear to be directly mechanistically linked to lysogeny. Instead, it indirectly measures a factor thought to favor lysogeny in an ecosystem. Additionally, this method suffers from the same issues regarding ‘fake virus particles’ as mentioned above.

Several studies have shown a decrease in VMR at high host densities ([Knowles et al., 2016](https://doi.org/10.1038/nature17193); [Wigington et al., 2016](https://doi.org/10.1038/nmicrobiol.2015.24)). A mechanism involving the increased prevalence of lysogeny at high host densities has been proposed to explain this phenomenon. However, this proposed link between the decrease in VMR and an increased prevalence of lysogeny has been disputed ([Weitz et al., 2017](https://doi.org/10.1038/nature23295)).

**Genomics (and other ‘omics’)**

Phages may be identified by sequencing their genomes, whether they exist as free phages or are integrated into a bacterial genome. Based on this principle, genomics may be used in various ways to identify genetic indicators of lysogeny. In phage ecology, metagenomics are typically used to identify these genetic indicators of lysogeny. Briefly, metagenomics involves the sequencing of all DNA found in an environmental sample, followed by bioinformatic analysis of these sequences ([Hoyles & Swann, 2019](https://doi.org/10.1016/b978-0-12-812293-8.00018-9)).

**Temperate Markers in Free Phage Genomes**

*Additional Definitions:*

* *Virulence factor:* Phage-encoded genes promoting increased pathogenicity of the phage’s host ([Boyd, 2012](https://doi.org/10.1016/b978-0-12-394621-8.00014-5)). Thus, a temperate phage carrying a virulence factor will provide its host bacterium with genes which may improve the host’s fitness, such as genes relating to toxin production, survival in harsh environments, or ability to infect other cells ([Boyd, 2012](https://doi.org/10.1016/b978-0-12-394621-8.00014-5), [Touchon et al., 2016](https://doi.org/10.1038/ismej.2016.47)). Not to be confused with virulent phage.
* *Integrase:* Enzyme used by temperate phages to integrate into bacterial genome; also used to excise prophages from bacterial genome ([Groth & Calos, 2004](https://doi.org/10.1016/j.jmb.2003.09.082)).
* *Excisionase:* Enzyme used by temperate phages (in prophage form) to excise itself from bacterial genome ([Cho et al., 2002](https://doi.org/10.1128/jb.184.18.5200-5203.2002)).
* *CI repressor:* Repressor responsible for maintaining lysogenic state in phage lambda ([Gandon, 2016](https://doi.org/10.1016/j.tim.2016.02.008)).

The genome of a phage determines whether or not it is capable of lysogeny. Measuring the presence of lysogeny-related genes in free phage metagenomes may reveal **the existence and relative proportions of free temperate phages.** However, there exist several theoretical risks with this approach. Obligately lytic phages may be descended from temperate phages, and may show genomic similarity. Therefore, metagenomic methods designed to detect free temperate phages may also detect obligately lytic phages, which cannot undergo the lysogenic cycle. On a more fundamental level, the presence of temperate genes in the environment does not necessarily indicate that these genes are being used; an increase in temperate genes in an environment may also indicate that the environment promotes the lytic mode of replication among temperate phages or the induction of prophages ([Weitz et al., 2017](https://doi.org/10.1038/nature23295)).

Many metagenomic studies in viral ecology come from aquatic ecosystems, such as freshwater or marine biomes. The following procedure is approximately representative of the data collection steps in many of these studies ([Angly et al., 2006](https://doi.org/10.1371/journal.pbio.0040368); [Breitbart et al., 2002](https://doi.org/10.1073/pnas.202488399); [Cassman et al., 2012](https://doi.org/10.1111/j.1462-2920.2012.02891.x), [Dinsdale et al., 2008](https://doi.org/10.1371/journal.pone.0001584); [Luo et al., 2017](https://doi.org/10.1128/mbio.01903-17); [Mende et al., 2017](https://doi.org/10.1038/s41564-017-0008-3)).

The protocol published by [Casas & Rohwer](https://doi.org/10.1016/s0076-6879(06)21020-6) is representative of most protocols which have been reviewed. In this study, samples of water were collected from a desired ecosystem. The samples were then filtered with a filter designed to remove small microbes such as bacteria, leaving only viruses. Most of these filters focus on viruses smaller than 0.22 µm in diameter, a fraction which contains most of the viral particles ([Williamson, Rusch et al., 2008](https://doi.org/10.1371/journal.pone.0001456)). It is important to note that this step could occur before or after a step meant to increase the concentrations of microbes or phages in the sample. Phages were then typically concentrated and purified with cesium chloride density-dependent centrifugation, a process designed to remove free DNA and cellular material. Nucleic acids were then extracted from the viruses using a method involving the lysis of the viruses, then amplified using commercial amplification kits. Various sequencing methods (mostly pyrosequencing) were used to sequence viral genomes, but all constituted a ‘shotgun’ sequencing approach ([Petrosino et al., 2009](https://doi.org/10.1373/clinchem.2008.107565)). Short fragments of nucleic acids (‘reads’) were sequenced, and either assembled into a full contiguous (‘contig’) sequence using bioinformatic tools, or were analyzed without assembly. For contig assembly, contigs were assembled based on read sequence overlaps. Bioinformatic comparison tools such as BLAST, which were typically used to understand the metagenomic genomes, are typically based on sequence similarity to known sequences. However, a known limitation with metagenomics as a whole is that it is difficult to determine whether or not a contig belongs to a single organism ([Quince et al., 2017](https://doi.org/10.1038/nbt.3935)). A contig incorporating sequences from multiple organisms is known as a ‘chimera’, which may be avoided in several ways. For example, some metagenomic studies do not attempt to assemble contigs, instead analyzing each read separately ([Cassman et al., 2012](https://doi.org/10.1111/j.1462-2920.2012.02891.x)).

Regardless of these challenges, the measurement of temperate phage markers is widely used to study phage ecology. Some common genes used to determine the presence of temperate phage are genes directly associated with the temperate life cycle, such as genes coding for integrase, excisionase, and the lambda CI repressor ([Luo et al., 2017](https://doi.org/10.1128/mbio.01903-17)). One study combining the metagenomic and the forced induction approaches showed that the presence of free temperate phage as measured by integrase levels corresponded with an increased proportion of lysogens as measured by the forced induction method, possibly alleviating some theoretical concerns with the use of integrase as a proxy for lysogeny ([McDaniel et al., 2008](https://doi.org/10.1371/journal.pone.0003263)). Similarly, the presence of temperate phage may also be measured through the presence of genes coding for virulence factors. It is thought that temperate phages encode virulence factors as a way of benefitting hosts which would benefit from such genes, as phages benefit more from lysogenizing such fast-growing pathogenic bacteria compared to slow-growing, free living bacteria ([Touchon et al., 2016](https://doi.org/10.1038/ismej.2016.47)). Additionally, phylogeny has also been used to identify members of a known clade of temperate phage, based on the evolution of a conserved viral gene ([Schmidt et al., 2013](https://doi.org/10.1038/ismej.2013.124)).

Moving beyond the examination of single gene markers, entire phage genomes or metagenomes have been analyzed for their similarity to known temperate phages to determine the presence of temperate phage ([Dinsdale et al., 2008](https://doi.org/10.1371/journal.pone.0001584)). One unique challenge with this approach is that it may be unable to detect temperate phages dissimilar to currently known phages. This may be problematic, as it has been shown that prophage communities carry many unique sequences which do not appear in known genomic databases ([McDaniel et al., 2013](https://doi.org/10.1111/1462-2920.12184)).

**Genomic Diversity of Phages**

It has been observed that there is **a decrease in free phage genomic diversity** in extreme environments compared to non-extreme environments, which has lead to the hypothesis that the decrease in phage diversity in extreme environments is related to the lowered diversity of hosts capable of surviving extreme conditions, coupled with the high host specificity of phages ([Cassman et al., 2012](https://doi.org/10.1111/j.1462-2920.2012.02891.x); [Williamson, Cary et al., 2008](https://doi.org/10.1038/ismej.2008.73)). It is also hypothesized that lysogeny is favored in extreme environments, allowing the transmission of beneficial phenotypes for the hosts, while reducing the likelihood that the phage will be destroyed outside the bacterium. Thus, one hypothesis suggests that there exists a correlation between lysogeny and lowered free phage diversity mediated by extreme environments. Additionally, while not strictly related to the diversity of free phages, other studies have shown that a community of induced prophages also exhibited reduced genomic diversity compared to free phages from the same site, further suggesting a correlation between lysogeny and phage diversity ([McDaniel et al., 2013](https://doi.org/10.1111/1462-2920.12184)).

This method correlates decreased genomic diversity to increased prevalence of lysogeny, but does not establish a mechanistic link between the two. If the correlation between lysogeny and genomic diversity is indeed mediated by the harshness of the environment, a reduction in VMR would also be expected. Studies measuring phage genomic diversity use the previously described metagenomic methods for isolating and sequencing phages.

**Prophages in Bacterial Genomes**

An infecting temperate phage undergoing the lysogenic cycle will incorporate its genome into the bacterial genome. By identifying phage genomes within bacterial genomes, we may identify **prophage sequences within sequenced bacterial genomes.** Using current bioinformatic tools, one may even differentiate between active prophages and prophage-like sequences (cryptic prophages). Prophage-finding tools work by scanning a bacterial genome for sequence similarity with known prophages ([Lima-Mendez et al., 2008](https://doi.org/10.1093/bioinformatics/btn043)).

Several studies have utilized this technique to identify prophages within published data, whether obtained from metagenomic or single-cell sequencing. For example, one study was able to quantify the relative abundance of lysogenic infections from human gut metagenomes based on the presence of prophages within bacterial genomes. These microbial metagenomes may be obtained through a similar method to viral metagenomes, but by selecting the microbial fraction with diameters between 0.22 µm-1.6 µm ([Williamson, Rusch et al., 2008](https://doi.org/10.1371/journal.pone.0001456)).

**Lysogeny Related Gene Activity in Bacteria**

The activity of lysogeny-related genes can be measured through the relative numbers of gene transcripts. Therefore, when observing bacteria, the activity of lysogeny-associated gene transcripts likely indicates the presence of **temperate phages undergoing activities related to the lysogenic cycle within the bacterial population.** It is also possible to measure the activity of the lytic cycle in a similar fashion through measuring the activity of lytic cycle-associated gene products. For example, one study found that the activity of the lysogeny-related genes transposase and recombinase were significantly negatively correlated with the activity of the gene coding for a viral tail sheath, a gene associated with the lytic cycle ([Stough et al., 2017](https://doi.org/10.1371/journal.pone.0184146)). Transcriptomes may be characterized using methods similar to the metagenomic methods described above ([Stough et al., 2017](https://doi.org/10.1371/journal.pone.0184146), [Santiago-Rodriguez et al., 2015](https://doi.org/10.1186/s12864-015-1781-0)). Microbial samples were taken from an ecosystem, followed by the extraction of all the RNA in the sample. Then, rRNA was removed and the remaining mRNA (and probably tRNA) was sequenced using shotgun sequencing. However, there may still exist theoretical problems with using this approach to measure lysogeny, related to temperate genes with multiple functions such as integrase. For example, integrase may be used to both initiate and end the lysogenic cycle, and therefore, measuring integrase activity may be insufficient to determine whether or not lysogeny is favored in a system ([Cho et al., 2002](https://doi.org/10.1128/jb.184.18.5200-5203.2002)).

**Polymerase Chain Reaction**

*Additional Definitions:*

* *Polymerase chain reaction (PCR):* A method of replicating any sequences bracketed between two given primer sequences ([Karcher, 1995](https://doi.org/10.1016/b978-012397720-5.50040-2)).

PCR is capable of detecting any sequence between two primers. One simple use of this idea is to use PCR to detect temperate phage by detecting a conserved temperate viral gene, and also using PCR to detect bacteria by detecting a conserved bacterial gene. One study diluted a bacterial sample from the environment such that, on average, only one bacteria resided in each well of a multi-well plate, then used the PCR method to determine which wells contained both a bacterium and a virus associated with the bacterium ([Tadmor et al., 2011](https://doi.org/10.1126/science.1200758)). Using this method, the researchers identified **novel temperate phage-host pairs** by observing phage-host pairs which were consistently colocalized. One limitation of the method in the context of measuring lysogeny is that colocalizations need not be associated with lysogeny (for example, even if amplifying temperate genes, an obligately lytic phage may still carry temperate genes and become associated with its host through the lytic cycle or through binding).

However, more commonly, PCR is used to detect **the presence of free temperate phage in a sample.** In some studies, PCR is used to amplify a conserved temperate gene, therefore confirming the presence of temperate phage. However, the same theoretical limitations as viral metagenomics apply; the presence of temperate phage does not necessarily confirm lysogenic activity. Another use of PCR is to detect the **successful integration of a phage into a bacterial genome** ([Askora et al., 2017](https://doi.org/10.3389/fmicb.2017.02212)). If the insertion location of the phage is known and the phage genome is known, a PCR may be designed with a primer with a phage sequence and a primer with a bacterial sequence. This test may be used to determine the presence of lysogens in a target population, as only a lysogen will contain both a phage and bacterial genome on the same strand of DNA. However, this method is limited to single phage-host pairs with known integration sites, bacterial genomes, and viral genomes.

**Subcellular Visualization Using Fluorescence**

Some factors leading to lysogeny, such as the number of coinfecting phages in a bacterium, depend on phage decisions at a subcellular level ([Gandon, 2016](https://doi.org/10.1016/j.tim.2016.02.008)). To study phage interactions on a subcellular level, fluorescence-based methods have been developed to **resolve the location of phages at a subcellular level**.

One study genetically modified phages to express capsid proteins attached to fluorescent proteins, allowing any generated phages to be tracked using fluorescence microscopy ([Zeng et al., 2010](https://doi.org/10.1016/j.cell.2010.03.034)). These phages were also made to express a different fluorescent protein under control of the same viral promoter controlling lysogeny, allowing the tracking of lysogenic bacteria. Together, these measures allowed accurate tracking of the life cycle of a phage on an intracellular level, for example, by determining the probability of lysogeny due to specific numbers of coinfecting phages. Another study used a method based on fluorescence in-situ hybridization to visualize the intracellular locations of phages and host ribosomes ([Allers et al., 2013](https://doi.org/10.1111/1462-2920.12100)). One DNA probe was made to hybridize to viral genes, while another was made to hybridize to host ribosomal RNA; after several other steps, the nucleic acid surrounding each probe was made to fluoresce. Thus, the relative location of bacteria in space could be determined through the probe attached to the ribosomes, while the relative location of the phages in space could be determined through the phage sequence probe. One proposed usage of this system to study temperate phage was to allow researchers to monitor the induction of a prophage and its transition to the lytic cycle.

One limitation is that these methods are only useful when the phage genome (or at least some phage genes) are known. Additionally, although probe sequences are not difficult to obtain, each probe sequence or genetically modified phage is only applicable to study a limited subset of phage-host pairs, rather than being applicable to all phages and hosts in an ecosystem ([Allers et al., 2013](https://doi.org/10.1111/1462-2920.12100)).

**Conclusion**

Many methods are used to study lysogeny in many distinct ways, from the single-cell scale to the ecological scale. Each method measures lysogeny in a different way, with differing levels of being mechanistically linked to lysogeny. Some methods, such as inducing the lytic cycle in lysogens, are clearly theoretically valid. Other measurements, such as measurements of the VMR, are not so clearly mechanistically linked to lysogeny. Additionally, the validity of the common approach of measuring extracellular markers of temperate phage has been called into question ([Weitz et al., 2017](https://doi.org/10.1038/nature23295)). Despite potential issues with several of the measurements, these measures have been used to advance the study of lysogeny and phage ecology as a whole.

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