

R i s k A s s e s s m e n t :

REPROGRAMMATOR

November 2014

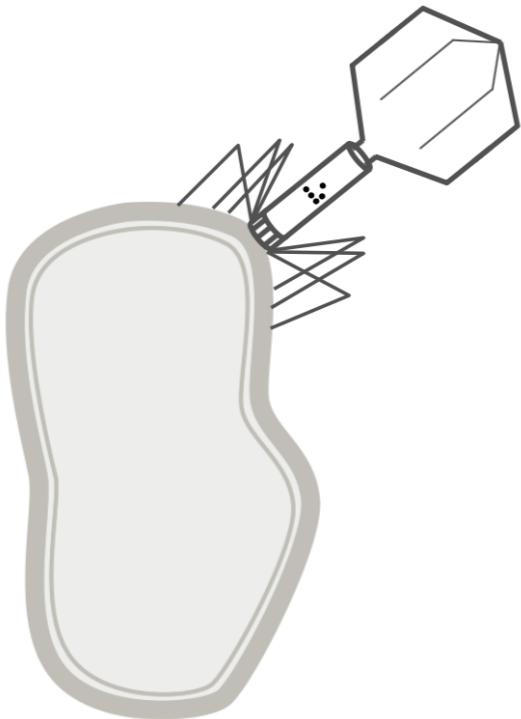
OVERVIEW

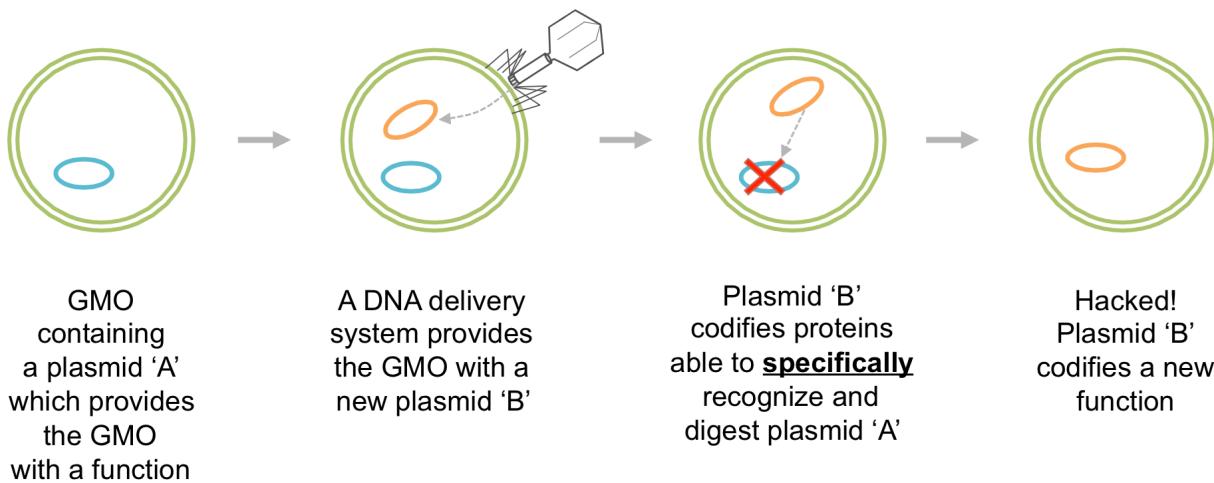
This project allows you to program a microorganism in a way that meets a function de novo, and also allows to "reprogram" the genetic program of the organism in situ, so that you can enter an agent to the environment once it has fulfilled its function reprogram either to stop the current program, change a new one or get rid of said microorganism specifically altogether.

The project makes use of bacteriophages to detect the biological agent of interest without affecting other natural organisms in the environment, and avoiding the use of dangerous chemicals that harm the ecosystem. Aims to generate a further control scheme of biological agents introduced into the environment as a safety means to allow the use of biological machines. Additionally, it is expected that the strategy used allowed to act in natural pathogens (bacteria, virus, etc...) And thus make them non pathogens when you change their genetic programmation.

Bacteriophages are bacterial viruses and consist of a single- or double-stranded DNA or RNA protected by a protein capsid. They are able to infect bacteria by injecting their nucleic acids inside the host.

A evaluation of the properties of the bacteriophage P1 and the organisms used in this project is required to implement adequate biocontainment and measures to support the human health and the environment.





BACTERIOPHAGE P1

Organism description

Bacteriophage P1 has been widely used to construct new bacterial strains and was used extensively to map the *Escherichia coli* chromosome. P1 has served as a model organism for different aspects of phage and biology such as DNA restriction modification, site-specific recombination and plasmid replication (Łobocka MB, et al 2004).

Understanding phage genetics allowed the development of major basic tools still in use in recombinant DNA technologies. Phage P1 exhibits the classical bacteriophage morphology with an icosahedral head, 220nm long inflexible tail with a complete tube surrounded by a contractile sheath, baseplate and six kinked tail fibers. The icosahedral head contains the phage genome. A variable part (encoded by an invertible segment of P1 DNA) of the tail fibers (1 to 2nm thick) determines the specificity of P1 adsorption on different hosts.

P1, like lambda, made its mark early in molecular biology. The significant capacity of P1 for mediating generalized transduction led promptly to P1 becoming a workhorse of genetic exchange among strains of *E. coli*, a role it is still playing today. Moreover, because P1 can package slightly more than twice as much DNA as can, and packaging can be efficiently carried out *in vitro*, P1-based vectors are now in common use for cloning and *in vitro* packaging of eukaryotic DNA. The recognition that P1 is maintained as a plasmid prophage led to the identification of its plasmid maintenance functions (Łobocka MB, et al 2004).

Properties

Plasmid 40784: BBa_J72113-BBa_J72152

- Gene/insert name: Phagemid + GFP
- Vector backbone: p15a, CamR
- Vector type: Bacterial Expression, SynBio
- Bacterial resistance(s): Chloramphenicol
- Growth strain(s): DH10B
- Growth temperature (°C): 37°C
- High or low copy: High Copy
- Terms and Licenses: UBMTA Ancillary Agreement for Plasmids Containing FP Materials.
- Comments: For detailed composition information, look up the backbone (BBa_J72113) and insert (BBa_J72152) on the Registry of Standard Biological Parts. There is a G insert at 5860 of depositor's seq, in between the "phagemid" and GFP, T301C, T459C, these do not effect function. Addgene has sequenced a portion of this plasmid for verification. Full plasmid sequence is available only if provided by the depositing laboratory.

Plasmid 40780: BBa_J72110-BBa_J72103

- Gene/insert name: AraC-pBad + coi + cin + repL + pacA
- Insert size: 4722
- Vector backbone: p15a, AmpR, CamR
- Vector type: Bacterial Expression, SynBio; Phagemid
- Bacterial resistance(s): Ampicillin
- Growth strain(s): DH10B
- Growth temperature (°C): 37°C
- High or low copy: High Copy
- Terms and Licenses: UBMTA Ancillary Agreement for Plasmids Containing FP Materials.
- Comments: For detailed composition information, look up the backbone (BBa_J72110) and insert (BBa_J72103) on the Registry of Standard Biological Parts. T459C and T301C in AraC, C4096T, deletion T4313, C4425T, G insert at 4912, do not effect function. Addgene has sequenced a portion of this plasmid for verification. Full plasmid sequence is available only if provided by the depositing laboratory.

Potential risks

Although most bacteriophages do not represent a threat to human health (unless they are carrying virulence factors), the use of recombinant viral particles in some instances might raise some biosafety concerns by bringing and potentially disseminating new genetic traits among bacterial populations. Aspects that should be considered are the expected survival of the phage, its multiplication and dissemination in the identified ecosystems, and the

anticipated interaction among the phage and the organisms likely to be present in the exposed ecosystems.

The genetic information encoded by the bacteriophage, its ability to transfer its genome among bacterial hosts, its host spectrum as well as its persistence in the environment are properties that should be assessed. From a biosafety point of view, the introduction of new genetic material into the bacterial gene pool may have positive, negative, or neutral outcomes depending on the genetic marker introduced; thus, the nature of this genetic marker is of great importance.

The survival of a bacteriophage outside a host is extremely variable and depends on the nature of the phage itself. It is also highly influenced by surrounding environmental conditions, such as pH, ionic strength, temperature, soil structure, adsorption property, or sunlight.

For the bacteriophage P1 to survive in the environment, they need to infect susceptible bacteria hosts to replicate and propagate themselves. The host range of a bacteriophage, defined by which bacterial strains can be infected, depends on the host cell surface receptor (proteins, lipopolysaccharide, or other cell surface components) recognized by this phage. Many phages are known to be highly specific for their receptors and are therefore characterized by a narrow host range, limiting their infectivity to a single species or to specific bacterial strains within a species.

Phages are obligate parasites, and their use will inevitably involve the manipulation of their bacterial hosts. Consequently, assessing the risk of the activity necessitates the evaluation of the susceptible bacteria involved. Risks associated with their manipulation depend mainly on the nature of the inserted genetic material and the bacterial hosts used to propagate these vectors and should be assessed on a case-by-case basis. In this laboratory where we use non-pathogenic *E. coli* laboratory strains, in this case, both phage P1 and the bacterial strains do not represent any risk to human health with the correct manipulation techniques, and therefore the risk of such an activity could be considered negligible, also because the genetic material used in this project is not pathogenic, thus is not considered hazardous.

E. COLI - M1061 (DH10B DERIVATIVE)

Organism description

Escherichia coli is a member of the family Enterobacteriaceae and is a Gram-negative rod which can be motile by peritrichous flagella or nonmotile. *Escherichia* is also a facultative anaerobe which has both a respiratory and a fermentative type of metabolism, and commonly occurs in the intestinal tract of humans and other animals.

E. coli belongs to the taxonomic family known as Enterobacteriaceae, which is one of the best-defined groups of bacteria. The strain *E. coli* K-12 is a debilitated strain which does not normally colonize the human intestine. It has also been shown to survive poorly in the environment, has a history of safe commercial use, and is not known to have adverse effects on microorganisms or plants. Because of its wide use as a model organism in research in microbial genetics and physiology, and its use in industrial applications, *E. coli* K-12 is one of the most extensively studied microorganisms.

Properties

- Blue/white selection
- While DH10B has been classically reported to be galU galK, the preliminary genome sequence for DH10B indicates that DH10B (and by their lineage also TOP10 and any other MC1061 derivatives)
- Genome sequence indicates that DH10B is actually deoR⁺
- Streptomycin resistant
- Leucine auxotroph

Potential risks

E. coli K-12 is considered an exempt host system under the NIH Guidelines. This microorganism also falls under the Class 1 Containment under the European Federation of Biotechnology guidelines.

No data were available for assessing the release and survival specifically for fermentation facilities using *E. coli*. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms.

These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison. During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures.

The natural habitat of *E. coli* is the large bowel of mammals. However, *E. coli* K-12 has lost the ability to colonize the gut and cannot survive in the bowel for very long. The ability of *E. coli* to survive under environmental conditions is also limited.

E. coli K-12 is a well-studied bacterium which has been the subject of extensive research in microbial physiology and genetics and commercially exploited for a variety of industrial uses. The natural habitat of the parent species, *E. coli*, is the large bowel of mammals. *E. coli*

K-12 has a history of safe use. Its derivatives are currently used in a large number of industrial applications, including the production of specialty chemicals

E. coli K12 strains are not likely to pose a risk to human or animal health, to plants, or to other microorganisms.

Health: May cause eye irritation with susceptible persons. May cause skin irritation in susceptible persons. May be harmful by inhalation. May be harmful if swallowed.

In case of contact: Rinse with plenty of water. If symptoms arise, call a physician.

OTHER ORGANISMS

	DESCRIPTION	RISKS
Pseudomonas spp Risk group: 2	<p>The genus <i>Pseudomonas</i>, of the <i>Pseudomonadaceae</i> family, are motile gram-negative aerobic bacteria, with polar flagella which have an important role in pathogenicity. They are non-spore forming and can produce pigments, such as pyocyanine (green-blue) and pyorubrin (yellow-green) fluorescence</p>	<ul style="list-style-type: none">As opportunistic pathogens, <i>Pseudomonas</i> spp. often invades the host tissue and cause infection and bacteremia in immunocompromised hosts (e.g., HIV/AIDS, cystic fibrosis, bronchiectasis, and severe chronic obstructive pulmonary disease, burns, malignancy, or diabetes mellitus).The common site of infection is the lower respiratory tract, and severity ranges from colonization without immunological response to severe necrotizing bronchopneumonia; such severe infection in patients with cystic fibrosis is almost impossible to eradicate once established in the airwayPrimary hazards: Accidental parenteral inoculation, inhalation of infectious aerosols, accidental ingestion, or direct skin contactHost: humans, animals (wild, domestic, livestock), and plants (flora and fungi)
Clavibacter michiganensis Risk group: 2	<p><i>Clavibacter michiganensis</i> is an aerobic non-sporulating Gram-positive plant pathogenic actinomycete that currently constitutes the only species within the genus <i>Clavibacter</i>. <i>Clavibacter</i> is an unusual genera of phytopathogenic bacteria in that it is gram-positive and does not have a type 3 secretion system.</p>	<ul style="list-style-type: none">This organism is a plant pathogenThe bacterium is located in the xylem vessels where it can cause lysigenous cavities.Infected vessels contain viscous granular deposits, tyloses and bacterial masses.The pathogen also produces a toxic glycopeptide which has biological activity.The bacterium survives for a long time in plant debris, soil and on equipment and glasshouse structures.It probably does not survive long in soil per se.The main host of economic importance is tomatoes, but the pathogen has also been reported on other <i>Lycopersicon</i> spp. and on the wild plants <i>Solanum douglasii</i>, <i>S. nigrum</i> and <i>S. triflorum</i>.

	DESCRIPTION	RISKS
Serratia spp Risk group: 2	<p>Serratia spp. are chemoorganotrophic, facultative anaerobic bacteria with low nutritional requirements, and belong to the Enterobacteriaceae family. They possess peritrichous flagella that allow them to swim and swarm (with differentiation), and are ubiquitous in soil, water, and plant surfaces.</p>	<ul style="list-style-type: none">• Serratia spp. are opportunistic pathogens and are one of the ten most common causes of bacteremia in North America. They are responsible for a variety of infections, including bacteremia, pneumonia, intravenous catheter-associated infections, osteomyelitis, endocarditis, and, rarely, endogenous and exogenous endophthalmitis• Primary hazards: Accidental parenteral inoculation, droplets exposure of mucous membrane, infectious aerosols, and ingestion• Host: Plants and animals (including human) have been found to be hosts to the different Serratia spp
Bacillus subtilis Risk group: I	<p>The genus Bacillus consists of a large number of diverse, rod-shaped Gram positive (or positive only in early stages of growth) bacteria that are motile by peritrichous flagella and are aerobic. Members of the genus are capable of producing endospores that are highly resistant to unfavorable environment conditions</p> <p>Bacillus subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. B. subtilis produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling.</p> <p>B. subtilis is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids.</p>	<ul style="list-style-type: none">• B. subtilis is widely distributed throughout the environment, particularly in soil, air, and decomposing plant residue. It has shown a capacity to grow over a wide range of temperatures including that of the human body. However, B. subtilis does not appear to have any specialized attachment mechanisms typically found in organisms capable of colonizing humans• B. subtilis appears to have a low degree of virulence to humans. It does not produce significant quantities of extracellular enzymes or possess other virulence factors that would predispose it to cause infection.• B. subtilis is not considered to be a plant pathogen

POTENTIAL RISKS

Project: REPROGRAMMATOR

Our project arise safety issues normally associated with working with typical cloning strains of the bacteria of the genus *E. coli*, *Pseudomonas*, *Bacillus*, *Serratia* and *Clavibacter*. Thus, in regard to researcher safety, our project pose risks that are contemplated in a normal microbiology and molecular training for a BL-1 and BL-2 laboratories, for example spills, contamination or accidental ingestion. These bacteria have very low competitive advantages against wild-type microorganisms in the case of an accidental release. Appropriate handling measures will be also applied for genetically modified bacteria and materials contaminated with bacteria. For the use of chemical reagents and laboratory equipment we will follow the

biosecurity rules imposed by Microbiological and Biomedical Laboratories (HHS 2009) and the WHO.

We are the first line of defense for protecting ourselves, others in the lab, and the public from exposure to hazardous agents. Protection depends on good microbiological practices and the correct use of safety equipment i.e.; always using personal protective equipment in addition to safety glasses, lab coat, gloves, and the safety equipment such as sealed rotors to provide a high degree of protection for us from exposure to microbial aerosols and droplets.

As we know the bacteria of the genus *Pseudomonas*, *Serratia* and *Clavibacter* are classified in level 2 of biosafety level, but only *Serratia* and *Pseudomonas* are classified as human pathogens, and these are considered opportunistic pathogens, which can rule out direct infection. So the good laboratory practices will ensure our personal to stay safe and to avoid accidents of biocontainment.

Only the bacteria of the genus *Clavibacter* is known as infectious to plants, which in the event of a crash would compromise certain species. Through good laboratory procedures, none of the genetically modified bacteria should have a chance of being introduced into the environment. Regarding environmental release, the main concern might be possible horizontal gene transference and possible infections to human and some plants, however any of the strains used can be consider as pathogen for humans or animals or plants. So, the chances for malicious mis-use by other person/group are minimal or null.

At the moment our system does not have any in-built, designed feature meant to alleviate a contingency. However, we have been careful to take measures to avoid any accidental release. Actually, we are working in a system for the specific infection/programmation exclusive for the bacteria strains used in our project.

The project contemplate the use of bacteriophages in order to change the transgenes in bacteria and substitute the resident genetic program for new one with the possibility to change again and over and over as a new program can be generated and insert by phage infection. This project advantage could be potentially risked only if the bacteria/phage specificity can be overturned. Finally, the original program in the receptor bacteria must have specific sequences in order to be deleted by the new incoming program. This make very difficult to widely spread in the environment, as the natural genetic plasmids or genomes, lacks the specific sequence.

The main idea of our project is to develop a specific phage to programme some specific bacteria strains (with unique characteristics) and in this way the interaction of virus-bacteria is able and safe to program and re-programmate the bacteria.

LABORATORY

Workplace & Biocontainment

The use of sharp tools or needles that may cause accidental injuries, should be avoided whenever possible. Other practices, such as pipetting, vortexing, and centrifugation, that generate aerosols, thus increasing the probability of exposure and the risk associated to the activity, should be physically contained by using sealed rotors, capped tubes, or biosafety cabinet.

The examination of biological risks related to the use of bacteriophages and the measures taken to reduce these biological risks allow the determination of an adequate containment level to optimally protect human health and the environment against the identified risks.

Phages handled in laboratories that are engineered to present no biological risks to human health can be handled in BSL-1. However, the containment level required should be adapted depending on the bacterial strains used to propagate the bacteriophages.

Indeed, bacteriophages represent an important gene reservoir that can be transferred among bacteria, and dissemination of recombinant phages into the environment could have important impacts on the surrounding susceptible bacterial population. The main precaution that has to be taken to avoid such unwanted release consists of properly inactivating all biological wastes generated by the activity that may contain bacteriophages. Bacteriophage inactivation is also important to avoid their dissemination throughout the laboratory (Verheust C., et al 2010).

Cleaning efforts should be as thorough and systematic as possible (work surfaces and all equipment used) to remove any residual phages, and data indicating which disinfectant and inactivation treatment are effective should be readily available for all workers in the form of written standard operating procedures.

Furthermore, since phages can exist for a longer time in wet environments, spills should be immediately cleaned up and any humid area should be eliminated. Good sampling handling and storage techniques and waste management practices are also essential.

- Respect good microbiological practices.
- Use appropriate disinfectant before autoclaving non-disposable equipment.
- Clean working surfaces regularly with adequate disinfecting solution.
- Clean up any spill immediately with a validated disinfectant and disinfection method.
- Avoid procedures generating aerosols.
- Do not open flasks during cultivation unless absolutely necessary.

- Avoid moisture. Dry all glass and plastic materials carefully.

However, even though the probability of dissemination has been evaluated to be negligible, good microbiological practices including appropriate disinfection should be followed. The main biosafety measures recommended to prevent any toxicity or allergenicity towards the worker consist of wearing a lab coat and gloves. Sharp instruments should be avoided if possible.

Hazardous substances

NaOH

Ethidium Bromide

Glacial Acetic Acid

HCl

SAFETY PROPOSAL

Safety suggestions

Determine the specificity of the phage to the multiple strains of the genre *E. coli*, *Pseudomonas*, *Clavibacter*, *Serratia* and *Bacillus*.

Determination of the host range of the manipulated bacteriophage is therefore an important step in the risk assessment process to evaluate the probability of the phage's propagation in a particular environment and its potential role in global gene transfer. Phage P1 are generally specific to one type of bacteria, but this host-specificity can be changed or expanded to other bacterial species. Restriction/modification systems may also be important parameters that affect and limit the host range of a phage in some bacterial strains. One way to address this particular problem is by engineering phages with genomes that do not contain restriction sites recognized by the bacterial host.

PROTOCOL: Specificity of bacteriophage P1 on multiple bacteria strains and *E. coli* M1061

To determine the specificity of bacteriophage perform the following protocol:

- An aliquot of liquid culture to evaluate strains (*E. coli* K-12: Top10, DH10B, DH5a, DB3.1, *Pseudomonas* spp, *Clavibacter* spp, *Bacillus subtilis* B479, *Pseudomonas* syringe,

Serrate spp) the OD is measured between 0.2 and 0.4, after obtaining this O.D., two aliquots of 300 μ L of each strain in a 96-well plate will be arranged.

- To evaluate the susceptibility one of the aliquots were taken as a control and to the other aliquot add 50 μ L of the bacteriophage P1 to the different strains to be evaluated, incubate for 1.5 h at 150 rpm, at 37°C.
- Measure the O.D. strains every 30 minutes until the incubation time is completed.

Determine potential multiple targets for the recognition sites of TALEN

With the BLAST tool from NCBI we are able to compare the recognition sites of TALEN in multiple genomes, basically divided in groups: Bacteria, Eukarya, Mammals and Human.

TALEN: GGAGCCATCTGGCCGGGT - Bacteria			
Organism	Position	Chain	Identities
Lactobacillus rhamnosus	2728302 - 2728285	Negative	100%
Frankia sp.	8764568 - 8764589	Positive	95%
Cronobacter sakazakii	1579360 - 1579376	Negative	100%
Gluconacetobacter xylinus	1219616 - 1219634	Positive	95%

TALEN: GGAGCCATCTGGCCGGGT - Eukarya				
Organism	Gene	Position	Chain	Identities
Triticum aestivum		382403448 - 382403434	Negative	100%
Drosophila melanogaster		23292211 - 23292225	Positive	100%
Cyprinus carpio		14230 - 14246	Positive	100%

TALEN: GGAGCCATCTGGCCGGGT - Mammals				
Organism	Gene	Position	Chain	Identities
Papio anubis	Collagen	1022 - 1005	Negative	100%

TALEN: GGAGCCATCTGGCCGGGT - Mammals				
Callithrix jacchus	Neurotrophin 3 (NTF 3)	376 - 393	Positive	95%
Chlorocebus sabaeus	Neurotrophin 3 (NTF 3)	262 - 279	Positive	100%
Balaenoptera acutorostrata scammoni	Neurotrophin 3 (NTF 3)	65 - 82	Positive	100%
Macaca fascicularis	Neurotrophin 3 (NTF 3)	38 - 55	Positive	100%
Chinchilla lanigera	Regulatory glutamine/lysine-rich protein	527 - 510	Negative	100%
Mustela putorius furo	Neurotrophin 3 (NTF 3)	4 - 21	Positive	100%
Mus musculus BAC clone RP23-240C17		188304-188321	Positive	100%

TALEN: GGAGCCATCTGGCCGGGT - Human				
Organism	Gene	Position	Chain	Identities
Homo sapiens 12 BAC RPCI11-1038A11		37191 - 37174	Negative	100%
Homo sapiens	Neurotrophin-3 {5' region, promoter}	3219 - 3236	Positive	100%

Determine phage viability under various conditions of pH, temperature and medium

Some phages can survive outside their microbial hosts for long periods of time under certain circumstances and maintain their ability to infect bacterial hosts. The survival and persistence of manipulated phages in soil or in other environments should therefore be studied carefully to evaluate the extent of potential risks in case of release.

PROTOCOL: Determining the viability of bacteriophage P1 in various states of stress.

a) Stress of pH to determine the viability of pH stress

- Phage be in liquid LB medium adjusted to different pH ranges; acid 4-6, optimum 7 and basic 8-10, then incubate overnight at 37°C.
- After incubation of virus, count CFU of *E.coli* MC1061 and incubate at 37°C. This way you will be measured if pH stress influences the viability of the virus in the process of infection.

b) Temperature stress

- The phage grown in LB medium and incubated at different temperature ranges: (32°C to 42°C.) For overnight.
- After incubation of virus, count CFU of *E.coli* MC1061 and incubate at 37°C. This way you will be measured if pH stress influences the viability of the virus in the process of infection.

c) Nutrient stress

- The phage grown in LB medium to be incubated at 37°C.
- After incubation of virus, count CFU of *E.coli* MC1061, which are hatched in different media (LB agar, M9 agar, LB agar supplemented with glycerol), and incubate at 37°C. This will measure whether nutrient stress affects the viability of the virus in the process of infection.

References

Verheust C., Pauwels K., Mahillon J., Helinski DR. and Herman P. 2010. Contained use of bacteriophages: Risk Assessment and biosafety recommendations. Applied Biosafety - ABSA. Vol. 15, No. 1: 32-44.

Łobocka MB., Rose DJ., Plunkett G., et al. 2004. Genome of Bacteriophage P1. Journal of Bacteriology. Vol. 186. No. 21: 7032-7068.

E. coli - genotypes: http://openwetware.org/wiki/E._coli_genotypes#DH10B_.28Invitrogen.29

E. coli - Risk Assessment: http://epa.gov/biotech_rule/pubs/fra/fra004.htm

Safety sheet - *E. coli* M1061: https://www3.appliedbiosystems.com/cms/groups/public/documents/msds/cms_095943.pdf

Clavibacter ABSA - <http://www.absa.org/riskgroups/bacteriasearch.php?genus=Clavibacter>
Safety sheet - Clavibacter: https://www.eppo.int/QUARANTINE/bacteria/Clavibacter_m_michiganensis/CORBMI_ds.pdf

Pseudomonas spp - Risk Assessment: <http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/pseudomonas-spp-eng.php>

Serratia spp - Risk Assessment: <http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/serratia-spp-eng.php>

Bacillus subtilis - Risk Assessment: http://www.epa.gov/biotech_rule/pubs/fra/fra009.htm
MTA Ancillary Agreement: Ancillary Agreement for Plasmids Containing FP Materials: <https://www.addgene.org/terms/1047/>