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DESIGN, REALIZATION AND CHARACTERIZATION OF AUTOMATED MILLIFLUIDIC BIOREACTORS FOR INVESTIGATING THE MOLECULAR EVOLUTION OF LYTIC OR LYSOGENIC VECTOR PHAGES INFECTING ENGINEERED HOST E. COLI

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Alla mia famiglia,

a Marta

Un altro aspetto curioso della teoria dell'evoluzione
è che tutti pensano di capirla!

[J. L. Monod]

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Abstract

This document presents a bioreactor intended for maintaining desired biological conditions that maximize the evolution of lytic (T7) or lysogenic (M13) of cloning vectors phages.

To this aim, the manuscript highlights concepts related to the *Theory of Quasispecies* and the relationship between self-replication errors, and natural or artificial selection pressures on populations of viruses: the model of system evolution.

Maintaining populations of virus means to provide them a substrate in which they replicate. Complex and expensive prototypes of dedicated machines have already been developed by other research groups for growing continuous populations of bacteria. The bioreactor, object of this work, is part of the European project Evoprog (Jaramillo's Lab, University of Warwick), aiming to develop a *General-Purpose Programmable Machine Evolution on a Chip*. This device, using existing phage technology and synthetic regulation, is expected to produce biomolecules or biocomputational functionality two orders of magnitude faster than conventional techniques (PACE), while reducing consumable and overall costs.

This first protoype consists of one or more fermenters, where a bacterial cultuare is continuously grown under optimized conditions of concentration, pH and temperature; and a cellstat, a separate vessel, where only the replication of viruses happens. Both volumes are about few milliliters and properly interconnected to enable a continuous molecular screening experimental oriented.

The results of preliminary experiments to prove the reliability of the prototypes is presented here. The experiments conducted, indeed show the success of two viral continuous coltivations and the in vivo ricombination of engineerd lytic or lysogenic bacteriophages.

Limitations and future developments of the system are finally presented, in light of specific applications addressing the studies of a bacteriophage-based antimicrobial therapy.

Chapter 1

1. Assisted continuous evolution (ACE): theoretical and experimental aspects

The evolutionary mechanism can be described by a system of differential equations which shows how the selection of biomolecules is an intrinsic property of a population of individuals able to self-replicate. This demonstrates that, molecular evolution can be investigated through targeted experiments of ACE where the natural biological molecules, that do not perfectly adapt to technological or medical aims, can be improved repeating cycles of mutation, selection and amplification.

We present here some mathematical models and experimental strategies, shown in literature, for describing those processes and effects involved during molecular evolution.

1.1 Quasispecies Theory: relation among information, self-replication and selection of biomolecules

There are two most important classes of biological macromolecules in which the information is coded: polynucleotides and polypeptides [1]. These have a common assembly principle: both are products of a linear polymerization where various functional groups are bound on a regular and repetitive 'skeleton'. This means that the information is linked to matter that is steadily degrading, so information would be lost if it was not preserved by a constant replication. However, the reproduction is not a entirely accurate event: the errors that occur during the replication process generate changes on the meaning of original information. The information content has to be then evaluated by its new coded functionality.

[1] Polypeptides and polynucleotides: A polypeptide is a long, continuous, and unbranched peptide chain. Proteins consist of one or more polypeptides arranged in a biologically functional way, often bound to ligands such as coenzymes and cofactors. Peptides fall under the broad chemical classes of biological oligomers and polymers, alongside nucleic acids (DNA and RNA) made of nucleotides.

The structure of these polymers, indeed, allows to produce a variability expressed by the equation (1), which describes the number *N* of different heteropolymers, whose length is *v*, that can be constructed from a number of different monomers:

$$N=\mu^{v} \qquad (1)$$

Where: μ is 4 for the nucleic acids and 20 for natural proteins.

1.1.1 Self-replication concurs to the selection of molecular complexes and generation of Quasispecies

Nucleic acids are the only known natural biopolymers that can serve as a template for its own replication. The ability that allows the interactions between their complementary constituents is an intrinsic property of the molecular structure of nucleic acids. Therefore, the base pairing allows the synthesis of a unique complementary copy of each polynucleotide.

The kinetics of the autoreplication can be expressed by differential equations, whose simplest case is represented by the following equation:

$$\frac{dc_i(t)}{dt} = W_{ii}c_i(t) + \sum_{j\neq i} W_{ij}c_j\,(t) - \emptyset_i \qquad (2)$$

with *i,j*= 1, ... , *N*, and

$$W_{ii}=A_i\,Q_i\text{-}D_i\geq 0$$

where: *i,* variable index, representing all individual sequences in a population;
W_{ii}, speed rate for the correct replication of the species *i*;
A_i, speed rate for the formation of the species *i*;
Q_i, quality factor which represents the average probability for a faithful copy of the sequence. Then, $(1- Q_i)$ is the corresponding probability of abnormal reproduction;
D_i, speed rate for the decomposition of the species *i*;
W_{ij}, mutation coefficient, related to the formation probability of the sequence *i* due to an erroneous reproduction of the sequence *j*;
$c_i(t)$, concentration of the sequence *i* at time (t);
$\emptyset_i$, flow that allows the control through an input and an output in an open system.

The differential equations (2) describes the simultaneous growth in

competition of all individuals in a population and shows the following properties:

- mutability, wrong self-reproduction, appears when $Q_i < 1$ e $W_j \geq 0$;
- metabolism is linked to the irreversible formation and degradation of the molecules, values expressed here by $A_i c_i(t)$, $D_i c_i(t)$ e $\emptyset_i$.

In such a kind of system, the phenomenon of the competition takes place if all the individuals *i* present in a population are considered. We define then:

$x_i(t)$, number of relative population;

E_i, excess of productivity of the species *i*;

$\bar{E}(t)$, mean of the excess of productivity,

$$x_i(t) = \frac{c_i(t)}{\sum_j c_j(t)}$$

$$E_i = A_i - D_i$$

then equation (2) can be re-written as

$$\frac{dx_i(t)}{dt} = \left(W_{ii} - \bar{E}(t)\right)x_i(t) + \sum_{j \neq i} W_{ij} x_j(t) \qquad (3)$$

Equation (3) can be exactly solved thanks to a linear transformation [1] [2] [3]. It, thus results:

$$\frac{dy_i(t)}{dt} = (\Lambda_i - \bar{\Lambda}(t))y_i(t) \qquad (4)$$

where: Λ_i, eigenvalues and y_i, linear combinations of all the $x_i(t)$, are the weighted coefficients of the individual mutants. These, are the contributions of the individual mutants weighed;
$\bar{\Lambda}(t)$, the average value of the eigenvalues, corresponds to the average excessive productivity $\bar{E}(t)$ of the species *i*.

Therefore, according to equation (4),

- all the mutants distributions with $\Lambda_i < \bar{\Lambda}(t)$ die;
- all the mutant distributions with $\Lambda_i > \bar{\Lambda}(t)$are amplified, increasing $\bar{\Lambda}(t)$ up to reach its maximum speed value of replication A_{max}.

Increasing Λ_i, more and more sequences are removed from the competition so, only the mutant distribution that replicates more efficiently remains and this is the distribution with the greater eigenvalue $\Lambda_{\max}$.

We call this behavior selection and this is an intrinsic property of the autocatalytic reproduction of any population of individuals. Consequently, this means that selection does not apply to a single molecular species that replicates itself, but rather it applies to situations in which there are groups of mutant. Distribution of mutants with higher efficiency replication behaves as an individual species, therefore is called *Quasispecies* [4] [3]. The *Quasispecies*, in this way, replaces the previous wild strain (WT).

1.1.2 Wrong self-replication affects an assisted evolution process under a threshold error

During viral genome replication, errors are not statistically rare events. In these systems, many individual mutants appear quite commonly. The frequency of the production of a mutant by imprecise replication on the positions *i* of the genome long *v*, is expressed by the binomial theorem

$$Q_i = \binom{v}{i} q^{v-i}(1-q)^i \qquad (5)$$

Any nucleotide is incorporated in a certain position with a accuracy value expressed by a fidelity *q*, which is the geometric average of the individual (and position-dependent) error rates. Therefore, *(1- q)* represents the index of the mean error frequency. A frequency error of 0.1 is equivalent to a false incorporation (on average) every 10 positions.

On the other hand, the occurrence frequency of a certain mutant is also influenced by its replication rate. The *extremal principle* [2] (ie, results of a critical function), described by equation (4), considers this effect evaluating the presence of mutant distribution according to their fitness, or biological suitability: ability to reproduce themselves efficiently under specific environmental conditions. If the *Quasispecies* contains a master copy clearly dominant, and if we neglect the reverse mutation, it can be defined as

$$\sigma_o = \frac{A_0}{D_o + (\bar{A}_{i\neq 0} - \bar{D}_{i\neq 0})} \qquad (6)$$

[2] The Extremal Principle is a methodology, a useful problem solving tactics, where a solution of a problem is sought among possible candidates that satisfy some extreme conditions within the parameters of the problem.

σ_o correlates the master sequence productivity, its rate of degradation and over productivity average of all the competitors *i*. $\bar{A}_i \neq 0 e \bar{D}_i \neq 0$ are averages values calculated on all the mutants that form the distribution, except for the master copy.

With $\sigma_o Q_o \geq 1$ e $Q_o = q^{\nu}$, equation (6) leads to the following expression for the limit informational capacity

$$\nu_{max} \approx \frac{ln\, \sigma_o}{1-\bar{q}} \qquad (6a)$$

Therefore, equation (6a) defines the maximum length of sequence that can be stably reproduced under the influence of the selection.

It also introduces that a principle of a threshold error has to be considered. A distribution of mutants just below this threshold has the largest possible genetic variation, even though remaining (meta) stable. However, these distributions are not symmetrical. They include many individual "neutral" or "almost neutral" [3] species, as well as more than one, or even no one, dominant species.

As a result of the above considerations, the mean sequences representing the "center of gravity" of the distribution could not be the most suitable or more frequent species, defined: WT strain. Also, the overcoming of the error threshold leads instead, to an accumulation of errors and thus to a random distribution of sequences, with complete loss of information.

Therefore, "evolutionary tests" for the selection of advantageous mutants, in known environmental conditions, should occur along the periphery of the distribution of the *Quasispecies*, where those mutants appear with sufficiently large occurrence frequencies σ.

Several research groups has experimentally established that viruses behave as *Quasispecies* and operate near their error threshold [5] [6].

[3] Neutral species: a species' niche encompasses all of the factors it requires for growth and reproduction and how it impacts its environment. Neutral species have completely overlapped niches; they share the same niche and their fitness changes identically along an environmental gradient or niche axis. Greater niche differences correspond to less niche overlapping between species; species differ in their fitness at different points along an environmental gradient or niche axis.

1.1.3 Virus: natural models for molecular evolution experiments

The study of the mechanisms that mimic the natural evolution should be conducted with simple, self-replicating species that involves only a few biochemical reactions. For this reason, S. Spiegelman [7] (1971) chose the virus as first simple replicators. Viruses are infectious particles, made of nucleic acid enclosed in a protein capsid. They do not have its own metabolic apparatus and therefore they are not autonomous living organisms. After the infection of a host cell and the injection of viral nucleic acid in the cell, the viral genetic information is read and translated and compete with the cellular DNA. Often, this process is fatal to the host cell because all its resources are used in the synthesis of new viral particles. Moreover, viruses show a surprising efficient replication, with rapid generation time, high population density and, due to the high error rate in the replication, rapid adaptation to the selective constraints.

The viruses are hence, ideally the best for molecular evolution experiments and this is the reason of their wide use.

1.2 Experimental strategies: natural and artificial selection

This section exposes the principles about the strategies experimentally adopted for conducting experiments about the comprehension of the molecular evolution, based on known properties of a population.

- Natural selection promotes individuals with more efficient reproductive systems. Accordingly, their fitness is manifested by a substantial advantage in growth. Exploiting this strategies, mutants with defined properties emerge under the influence of a selective pressure.

Some experiments have been conducted and proved that, molecular replicators and bacteriophage [4] exposed to various environmental constraints,

[4] Bacteriophages: or phage, is a virus that through a contraction mechanism injects its nucleic acid in a given parasited bacterium. Once injected, the phage genome can follow two paths:
- Lytic cycle (typical of bacteriophage T7): use the replication machinery of the host to produce new phage particles, up to the volume of the outbreak, at which time the cell will disintegrate for lysis.
- Lysogenic cycle: however, the phage genome (usually λ or M13 phage) will integrate into a specific point in the bacterial chromosome, *attλ*. This integrated phage is called prophage, and

have resulted in species with increased replicative efficiency. Starting from bacteriophage Qβ, the viral infections have been studied under the influence of antibodies that neutralize the protein coating of the phage [8]. In another experiment small replicative RNA could compete with the viral genome for the replication operated by the polymerase encoded by the phage [9]. In both experiments some mutant phages evolved and were able to avoid the antiviral attack which they were subjected.

- Artificial and non organized selection, without competition, in which mutantas with any desired property are promoted by an external interference.

The artificial selection, unlike natural selection, is based on the precise knowledge of the desired property, and therefore requires a method to select large populations according to the characteristics that have been defined for selection. A typical selection process begins with an extensive random mutagenesis [5] of a clone that presents considerable similarities to the desired characteristics. The resulting (library) of the mutants is then selected by an assay suitable to detect promising mutant. This process is repeated on the selected mutants again and again until one or more optimal individuals are obtained. The feature of this artificial selection procedure is the separation between growth (or amplification) and selection: in contrast to the natural system, where the rate of optimal reproduction always plays an important role in the selection, here it is possible to choose any selection criterion. One of the first experiments in which this strategy has been applied was devised by L. C. Tuerk and Gold [10] (1991).
Natural and artificial selection can also be combined for the aims of the experiments investigated, where amplification, or self amplification, of the individuals is a common step.

every time the bacterial chromosome replicates, the genome of λ also replicates. A bacterium that contains a prophage is said "lysogenic".

[5] Mutagenesis is a process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation. It may occur spontaneously in nature, or as a result of exposure to mutagens. It can be achieved experimentally using laboratory procedures.

Chapter 2

2. State of the art: configuration, devices and technical solutions for experimental molecular evolution

Using a theoretical basis of molecular evolution, devices were designed and developed to perform molecular evolution experiments where the mutation, selection and amplification processes are kept stable and performed sequentially.

Such instruments are able to 'mimic' the evolution process and have been used to 'build' functional macromolecules, nucleic acids or proteins, for technological or medical applications.

2.1 Viral continuous culture: the cellstat system

The competition in a population of individuals emerges when the total number of individuals is maintained constant and it is described by the equations [3] and [4]. A population can be maintained numerically constant keeping constant all the necessary resources. Therefore, when a variant, which is more suitable than the original WT appears, the new population can overtake the original one reaching a higher total concentration of individuals. Indeed, the evolutionary events are usually detected with the emergence of faster replication species.

When maintaining constant conditions, the application of any new selective pressure increases the visibility of the fitness of some individuals.

It is possible, in principle, to obtain the continuous cultures of viruses for evolutionary studies. However, viruses are parasites, they do not grow on their own, but they need a substrate of host bacteria to replicate. These bacterial host cells can be produced in a continuous culture bioreactor fermenter.

The production of viruses can be realized by a device called cellstat and it can be divided in two mains units [11][12] (Fig. 1):

1) the fermenter is fed through a turbidimeter, a system that controls the

turbidity of the culture by a continuous measurement of absorbance and generates cultures of host cells with a constant physiological reproducibility in conditions of exponential growth phase;

2) cellstat reactors, where a steady flow of the host cell is infected with viruses, reaching steady state conditions and thereby producing constant amounts of phage, infected and not infected bacteria.

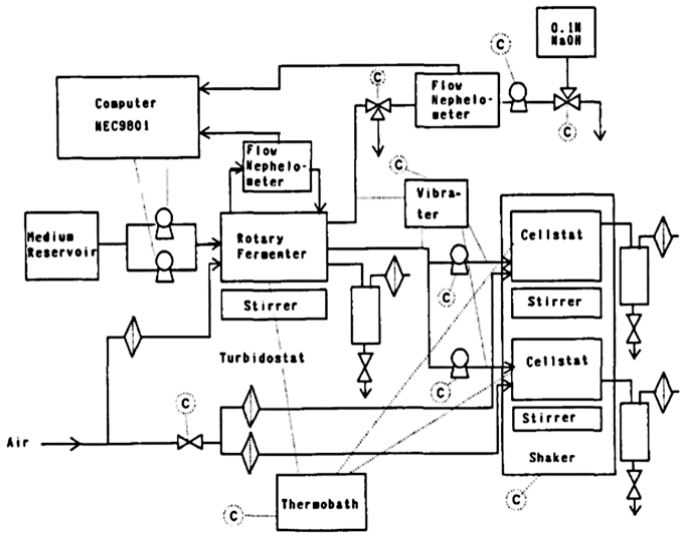


Figure 1: schematic diagram of the cellstat configuration system developed by *Husimi et al. Fig. 6 (1989)*

Maintaining separate bacterial and phage cultures avoids artifacts due to coevolution. If culture of the host and parasite are allowed into a single vessel this would inevitably lead to a bacterial population resistant to the virus, making the experiment meaningless. Rather, any bacteria potentially resistant to the infection has to be rapidly washed out from the cellstat system.

When a steady state is reached into the reactors, the evolution experiments can start, applying new and defined physical, chemical or biological selective pressures. The constraints applied to the system can lead to the natural selection of the phages, that in these conditions replicate more efficiently, and are therefore defined as the most suitable.

Thanks to this device it is possible to simulate viral infections of *superior* organisms for testing new antiviral strategies.

In a typical experiment conducted by Lindemann (1992), a phage Qβ has been exposed to the influence of monoclonal antibodies, directed against the

protein of the viral capsid, “simulating” then the immune response of a *superior* organism to a viral infection. This has allowed some phages of the population to escape the selective pressure, mutating the gene that encodes the capsid protein. The proteins analysis showed that these basic substitutions were expressed as altered antigenic epitopes on the viral surface. Later cultured mutants resistant to the antibody without selective pressure returned to be again a completely wild type phage.

In another experiment [13] a genetically modified phage (fd) for the expression of viral peptides on the surface [14] and containing a codified sequence for tetracycline resistance, was initially cultured into a cellstat without the antibiotic. As a result it lost the inserted gene encoding the tetracycline resistance. When, in a second experiment, the same phage was cultivated under the selective pressure of the antibiotic, the additional gene was stably inherited for several generations of phage.

Using continuous cultures of Qβ and *fd* phage allowed to demonstrate for the first time the mechanisms used by viruses to overcome antiviral therapy.

2.2 Molecular replicators evolution: the machine for mass-transfers

Experiments that exploit the cellstat configuration allowed the study of the competitive growth of living organisms. Such competition can also be studied in vitro, via molecular replicating species. The most used replicator in molecular evolutionary experiments is Qβ constituted by the replicase[6] of purified Qβ, which uses viral RNA as a template and nucleoside triphosphates as monomeric substrates. Spiegelman [15] (1965) was the first who, using this replicating system, applied constant conditions (i.e. similar to the cellstat). In the concept of transfers in series, a type of RNA is amplified to obtain a certain maximum concentration which is followed by a dilution of the molecular population in a new solution. In this way, the continuous alternation of phases of amplification and dilution simulates the characteristics described by the

[6] RNA replicase: is an enzyme that catalyzes the replication of RNA from an RNA template. This is in contrast to a typical DNA-dependent RNA polymerase, which catalyzes the transcription of RNA from a DNA template.

cellstat.

The molecular replicators are forced to evolve during the growth period, varying the growth conditions, for example, increasing the reaction temperature or adding substances. The products of the evolution can be analyzed after each transfer, providing information about the molecular mechanism of this process. If the external constraints are not applied, the speed of replication determines the composition of the population.

In general, it is important to execute evolution experiments in vitro under optimal conditions, in particular close to the error threshold, as suggested by the theory. Spiegelman's experiments were conducted far below the error threshold of Qβ replicase. The result is that in these experiments the evolutionary process was rather slow.

Then, a machine able to execute faster and automatically experiments of mass-transfers (Fig. 2) has been built. The reaction vessels and the sample transporter are made of silver coated with biological inert material (gold).

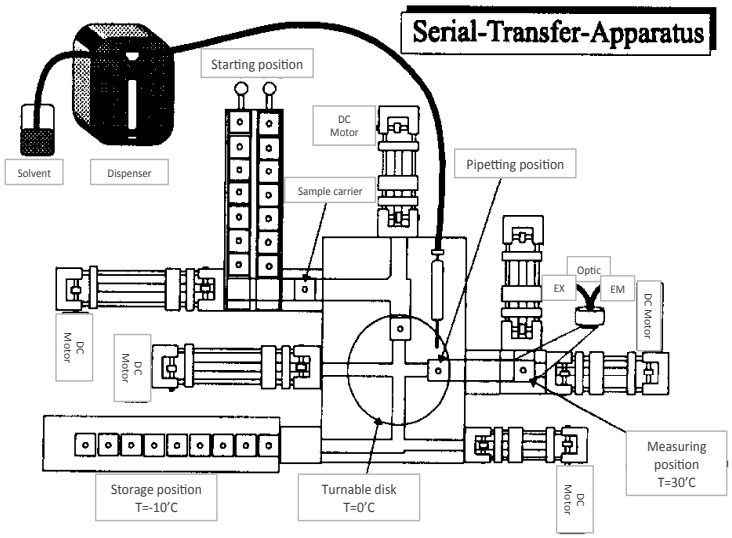


Figure 2: overview of the machine for automatic mass-transfers developed at the Max Planck Institute for Biophysical Chemistry in in Göttingen (Germany). *Strunk, Fig. 3 (1993)* .

Starting from the initial position, the samples are transported to the measuring position, which is thermostatically controlled to an optimal growth temperature. The amplification reaction is triggered by a temperature jump. The concentration of the nucleic acids continuously produced, can be directly

followed by a fiber optic fluorometer. Ethidium bromide, in the reaction solution, intercalates into nucleic acids with a concomitant increase of fluorescence intensity which is measured. The number of photons emitted is proportional to the concentration of the RNA, so the intensity of the observed fluorescence controls the automatic pipetting device (through a central processor). As soon as RNA exceeds the set maximum concentration, a robot pipette removes an aliquot of the solution and transfer it to the next samples transporter which, then, goes to the measuring station.

The evolution of a *Quasispecies* of RNA under the selective influence of the TI and a ribonucleases [7] was one of the first phenomena experimentally investigated with this device [16].

2.3 "Small" Quasispecies evolution: the multi-channel machine for mass-transfers

As demonstrated, the cellstat and the mass-transfers machine are the appropriate tools to investigate the molecular evolution. Both the machines can be used to study the Q*uasispecies* as global entities. Basically, a *Quasispecies* can be diluted up to the level of subpopulations, or even to the level of individual molecules, and each clone can be compartmentalized. In this way, the evolutionary adaptations of individual variants can be studied in parallel. Furthermore, the effect of an external constraints variety on mutants of a Q*uasispecies* can be observed. For this purpose a machine that allows to study in parallel 96, or even 960 individual replicating clones has been built.

This new robot enlarges the features of the previous machine for the mass-transfer, enabling evolutionary experiments assisted by the PCR [8].

The multi-channel machine for mass-transfers is made of three aluminum blocks, each one with 96 or 960 wells in which are placed the containers in

[7] Ribonucleases (Rnase) is a type of nuclease that catalyzes the degradation of RNA into smaller components.

[8] PCR: is a technology in molecular biology used to amplify a single copy or a few copies of of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

which the reactions take place. The temperatures of these three metal blocks are individually controlled by a computer. The solutions containing the molecular systems are loaded into plastic containers with cavities that are adapted to the wells of the metal holder. These cells are covered, air-tight, with another plastic sheet and they can be moved from one block to another at different temperatures as defined by the PCR. In these blocks temperature changes can be obtained in few seconds. As in the experiments executed in the 'single channel' machine, the concentrations of nucleic acids produced by PCR can be followed on line with a glass optical fibers fluorimeter of 960 channels. In addition, a robot is installed and it is capable of performing the necessary dilutions. The first experiments with this machine were conducted to investigate the influence of various chemicals on the amplification of DNA by the PCR. This machine can also be used to examine the artificial selection in the absence of competitive growth. Selections of new variants may be achieved by the simultaneous detection of 960 variants with assays that linked a fluorescent signal to a new or functionally optimized property, for example the incorporation of fluorescent analogs of nucleotides by a new mutant polymerase.

2.4 Evolutive events "imaging": the capillary reactor

One of the common features of all the machines described so far is that the reaction systems investigated with these devices display a spatial homogeneity. This property allows the observation of the competition among individuals. J.S. McCaskill described a machine with a one-dimensional reaction environment that, unlike the instruments described hitherto, would create a spatial inhomogeneity which is advantageous to obtain additional information on the replicating system [17]. This is due to the effect of both reaction and diffusion, which determines a constant speed of propagation, unlike the simple diffusion described by the typical report squared distance on time [18] :

$$v \approx 2\sqrt{kD} \qquad (7)$$

According to this equation, the expansion speed of a species v that grows

along a unidimensional “living space” simply depends on its rate of replication *k* and diffusion coefficient *D*. A molecular replicator then spreads like a “rambling cloud“.

The device that realizes this idea was introduced by McCaskill and Bauer (1993). It is a reactor consisting of 144 parallel capillaries filled with the replication system and an intercalating dye to view the new synthetized RNA molecules. In this way it is possible to control the spatial progress in this environment. The individual molecules of RNA are the source of the colonies growing exponentially. After a short period of time, these clones can expand through replication and they can spread only along the capillary, since their growth is limited in the other two dimensions by the outer walls. This constraint therefore determines a wave of concentration that proceeds with constant speed. A CCD camera installed above the area of the capillaries can capture images at regular time intervals. Series of these images can then be combined as space on time images (fig. 3) to show the differences of evolutionary events and, also, the variations of individual colonies into the reactor.

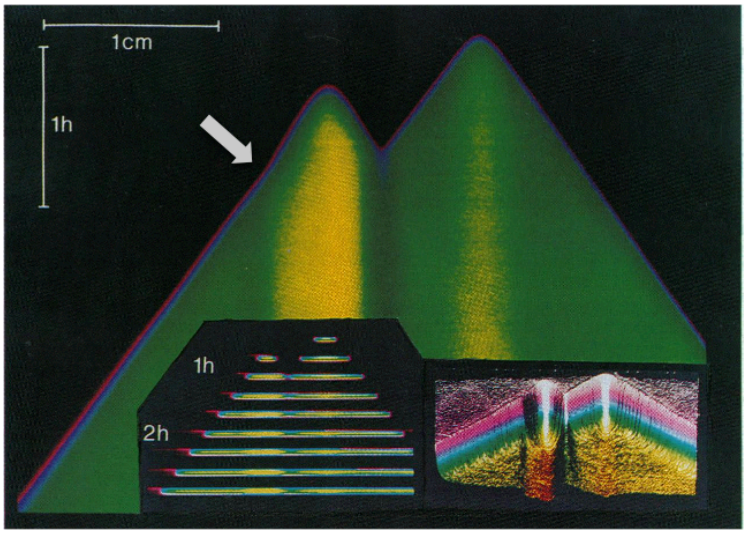


Figure 3: plotting of a wave front of rapidly increasing RNA concentration propagates at constant velocity to the left to and right of the positions where two single RNA seeded the replication. A sharp increase in the front velocity, mark by the arrow, reflects a single evolutionary change in the replication rate of the RNA. *Fig. 2 McCaskill and Bauer (1993).*

Thus, colonies originated from initial different molecules can be distinguished because their replication rate is matched to their expansion speed because clones with different rates of replication show, in the resulting images, different and characteristic gradients. Moreover, each evolutionary event associated with a change in the replicative rate alters the speed of advance of the front of wave that propagates, whose result is a change in the slope of the space-time curvature. The obvious advantage of the capillary reactor is the simplicity with which is possible to follow mutational events. After completing the experiment, the samples can be sequenced. In this way, the molecular strategies used to escape the constraints of the selection can be followed step by step.

Chapter 3

3. Materials and methods

Biological approaches and technical solutions for the development of this work are presented in this section.

The principles of the PACE (phage-assisted continuous evolution) technique and the innovations introduced by Jaramillo's Lab [School of Life Sciences, University of Warwick, FP7 Evoprog: General-Purpose Programmable Machine Evolution on a Chip] are equally decribed.

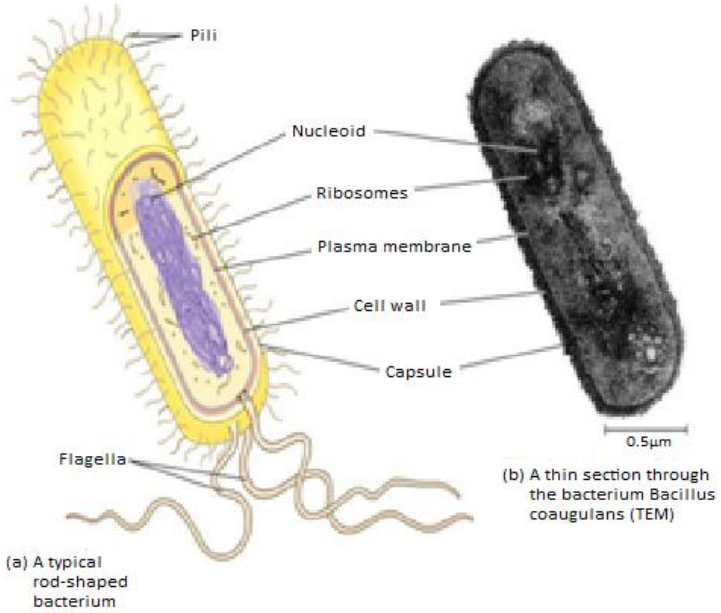
3.1 Biological parts

3.1.1 Escherichia coli bacteria: the "compartmental models for evolution"

The choice of Escherichia coli (E. coli) as a model system in biology derived from the extension of the general concept, at the genetic and molecular biology level, about uniformity of principles that govern living organisms. During the development of biochemical research in the thirties and forties, it became clear that all living organisms are formed, mostly, by the same chemical constituents. Also, mechanisms of production, transfer and use of energy, are largely mediated by ATP and the formation of high-energy phosphate bonds. With the development of molecular biology it became also clear that the storage and transfer of genetic information are essentially common to all living organisms.

E. coli, is a microorganism that grows easily in a simple culture media, containing any organic substance, as a source of carbon and energy, along with a few inorganic salts. Like many other bacteria, E. coli can grow within rather wide ranges of pH, osmotic pressure and oxygen tension. Finally it is attacked by numerous viruses.

All these features have made E. coli bacteria in practice, the model on which it is easier to conduct surveys and experiments (Fig. 4).



Figures 4: al schematic drawing of a typical rod-shaped bacterium and its cell structure; **bl** a TEM section of a E. coli.

E. coli: the growth

One of the reasons for the success of micro-organisms such as bacteria in the living world, is related to their rate of growth. An organism as E. coli, in favorable environmental conditions, is able to divide itself every twenty minutes: in other words its size doubles every twenty minutes and then divides by binary fission, in two equal halves. It follows that, to maintain this high rate of growth, Escherichia coli must have a correspondingly intense metabolism so the growth will be exponential until the environment will be suitable for the storage of the maximum rate of division (Fig. 5). This happens because E. coli has an high ratio of surface to volume. In fact, the speed of growth of an organism is typically limited by the rate of absorption of nutrients, such as oxygen, and the rapidity with which the catabolic products are eliminated. An increase to the maximum rate possible depends on various factors, such as temperature, pH, oxygen tension, osmotic pressure and nutrients in optimal concentration. An analysis of the necessary materials nutrient in Escherichia coli has highlighted that, although it can grow on a nutrient medium complex it

may also develop in a medium containing a single source of energy and carbon, together with other only inorganic nutrients; this is due to its biosynthetic ability to process all their chemical constituents starting from these simple constructive materials [19]. However, the study of the effect of changes in the type of nutrients, available during the growth, on the intracellular concentration of enzymes has led to an acquisition that was crucial for the development of molecular biology: some enzymes have no relationship with the environment, while others, called adaptive, are synthesized only in response to particular conditions. This ability to adapt to a great variety of different environmental conditions has been of considerable importance for biologists and it is the reason of a sharp distinction compared to many animal and plant cells that develop in very controlled environmental conditions.

A related topic with bacterial growth and that we will see later is the technique of continuous culture.

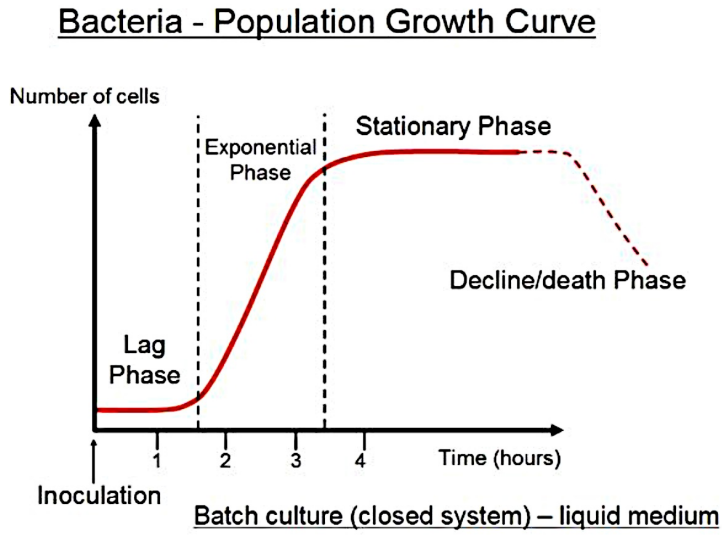


Figure 5: typical bacterial growth curve. Bacteria grown in a closed system (batch culture), exhibit these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Note the parameters of the x and y axes. Growth is expressed as change in the number viable cells vs time. Generation times are calculated during the exponential phase of growth. Time measurements are in hours for bacteria with short generation times.

E.coli: genome structure

E. coli genome is a circular double strand of DNA. Spontaneous mutations occur with a frequency similar to that found in other living organisms and, as E. coli is haploid, are easily identified [20].

Therefore, thanks to the ability of E. coli to oppose a broad spectrum of environmental conditions stemming from relevant selective pressures, it is possible to isolate with relative ease a large number of different mutant. An evolutionary process that, under natural condition,s is carried over longer times happens in a lab just in the space of one night. For example, if a bacterium sensitive to penicillin mutates developing a resistance to the antibiotic, a WT population can be incubated in a culture medium containing an appropriate concentration of penicillin, so that a sufficiently large number of microorganisms may develop a resistant phenotype.

Significant pressures of this type can be present in nature: antibiotic resistance in clinical settings is one obvious example. This effect could also inevitably affect the continuous culture of a single bacterial strain, when not properly setup.

The doubt related to the spontaneous mutation were clarified only after 1940, thanks to the discovery of the mechanisms responsible for genetic recombination. These techniques are commonly used for biological investigations that require the presence of mutants.

- Conjugation. J. Lederberg and EL Tatum in 1946 observed that if it were cultivated together two strains of Escherichia coli with different cultivation requirements, occurred a recombination of genetic determinants. This process requires physical contact between the two strains, one with the fertility factor (F^+) and the other without it (F^-). The conjugation is linked to the passage of DNA from the strain F^+ to that F^- (Fig. 6) [21].

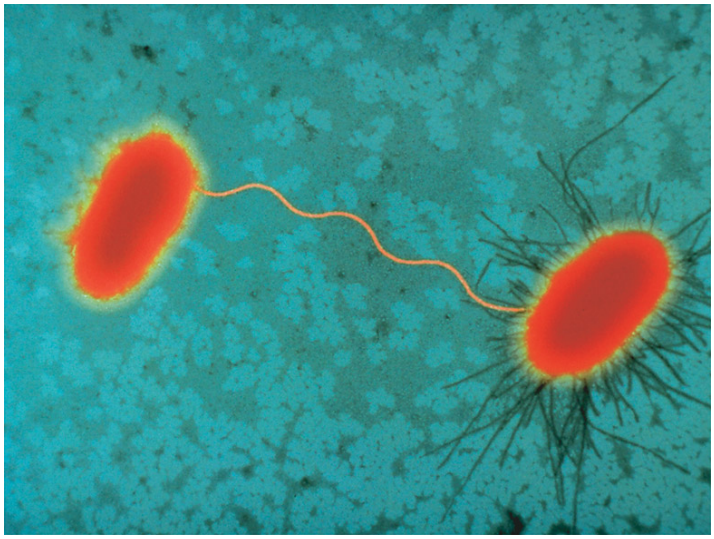


Figure 6: Sex pilus connecting two E. coli cells during bacterial conjugation; on the left a F^- cell is linked to a F^+ cell on the right.

- Transduction. The phage head normally contains the phage genome. However, a small percentage may accidentally include a segment of the genome from a previous host (in a variable number of genes, up to 150). When a phage of this type attacks an E. coli, the DNA segment penetrates the new host and integrate, according to the normal processes of recombination, in the chromosome of the new host [22].

If in continuous culture, this phenomenon could then cause the co-evolution of species.

- Transformation. With one of the most important biological research of this century, RJ Dubos and OT Avery showed that in the pneumococcus, causative agent of pneumonia, genes can be transferred from one cell to another by fragments of purified chromosomal DNA. This discovery was the first real proof that DNA is the repository of genetic information. The process is accomplished in the pneumococcus with a speed much greater than in other bacteria and microorganism such as E. coli. Such differences in undergoing the transformation seem mainly related to the problem of penetration of a large molecule, such as DNA in the cytoplasm and then into the nucleus, since the cell wall and the cytoplasmic membrane normally behave like a real barrier against such a transfer. It is therefore difficult to

assess the importance of transformation in nature [23].

Figure 7 resumes all the above presented procedures.

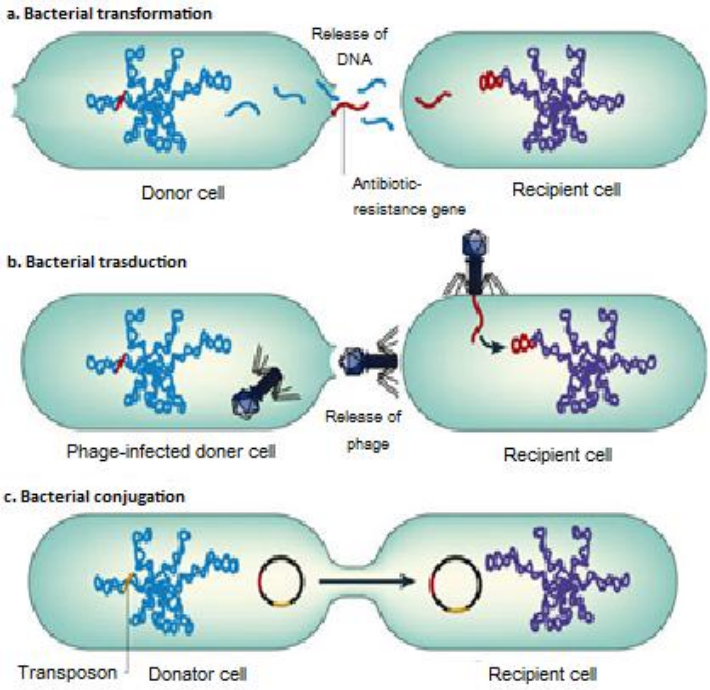


Figure 7: al Transformation occurs when naked DNA is released on lysis of an organism and is taken up by another organism. The antibiotic-resistance gene can be integrated into the chromosome or plasmid of the recipient cell. **bl** In transduction, antibiotic-resistance genes are transferred from one bacterium to another by means of bacteriophages and can be integrated into the chromosome of the recipient cell (lysogeny). **cl** Conjugation occurs by direct contact between two bacteria: plasmids form a mating bridge across the bacteria and DNA is exchanged, which can result in acquisition of antibiotic-resistance genes by the recipient cell. Transposons are sequences of DNA that carry their own recombination enzymes that allow for transposition from one location to another; transposons can also carry antibiotic-resistance genes. *Nature Reviews | Microbiology, 2006*.

- Another mechanism to transform E. coli, exploits plasmids located outside of the main chromosome. Plasmids are circular fragments of double-stranded DNA that reproduce themselves within the cell; they possess many properties of a small chromosome and contain genetic information to control their replication and to ensure segregation of their copies in each daughter cell at the time of cell division. They differ, however, from a chromosome as they are much smaller and are not essential for normal vitality of the cell. Sometimes a plasmid can be integrated into the main chromosome of the

bacterial cell, indicated by the term 'episome'. The F factor, involved in conjugation is an example of a plasmid. Factors of resistance to drugs may be present in plasmids rather than in the main chromosome: a single plasmid may encode resistance to many commonly used antibiotics.

In lab, plasmids can be transferred remarkably easily from one cell to another and they can cause the rapid spread of an antibiotic resistance within a bacterial population (Fig. 8).

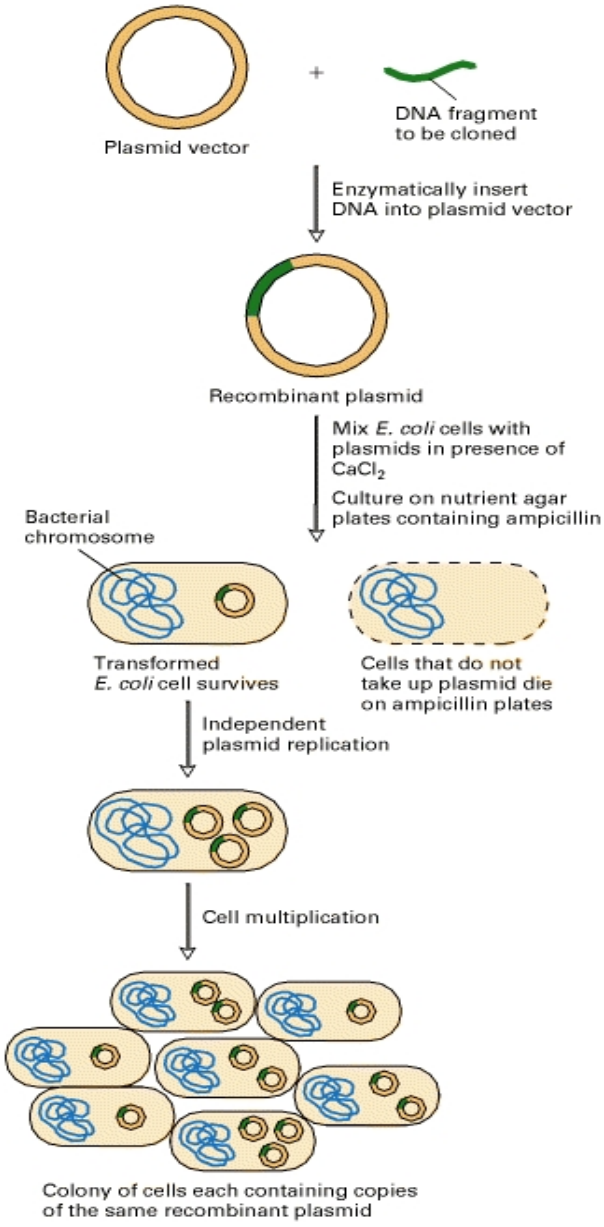


Figure 8: procedures of gene cloning into plasmid and transformation of E.coli bacteria.

3.1.2 Bacteriophages: the faster evolutionary replicator

During the second decade of the XX century FW Twort and FH d'Herelle gave a description of viral infections of the bacteria. They showed that bacterial cells can be infected and destroyed by filterable agents. These bacterial viruses were called bacteriophages or more simply phages. Although most phage present a more complex structure of the viruses of animals or plants, they have been studied deeply. The largest information on the mechanisms of phage reproduction and the influence of the host was obtained from viruses that infect E. coli: in particular from a group named T phages [24].

Phage: the structure

In the structure of the virus two main parts can be observed: a head and a tail. The head contains a single circular molecule of double-stranded DNA, or chromosome, whose molecular weight is 1.3×10^8 Dalton. It is surrounded by an icosahedral capsid protein structure of approximately 125 nm long and 81 nm wide. More precisely, each half of the head is constituted by a half icosahedron and the two halves are connected by a short hexagonal prism. The capsid is formed from individual protein subunits or capsomeres, whose repetitive structure determines the crystal symmetry of the head.

The tail of the phage is a complex structure of about 95 nm in length. Its innermost component is formed by tubule protein subunits, in helical arrangement and covered by a sheath, constituted by other subunits, which extends from a collar to a plate baseline and has the ability to contract to less than half of its extension. Even the collar and the basal plate are composed of other protein subunits and on the plate there are small pins which have six long and thin caudal fibers (Fig. 9).

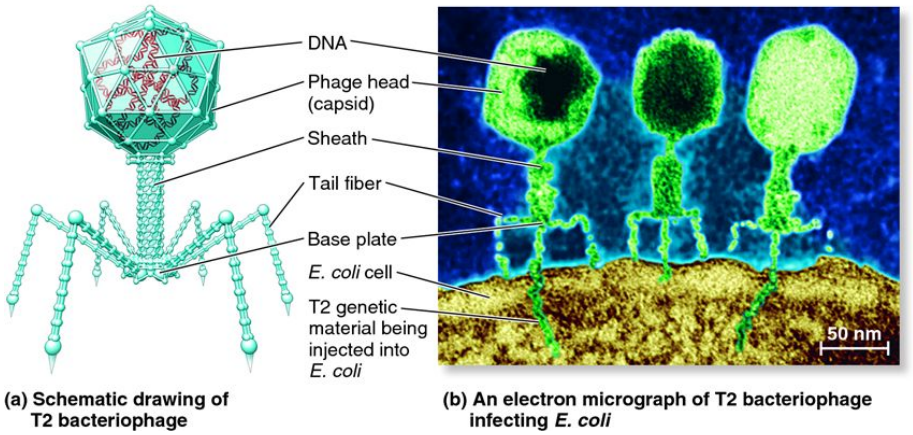


Figure 9: al schematic drawing of a lytic T2 bacteriophage; **b**l an electron micrograph of T2 bacteriophage infecting an E. coli cell.

Phage multiplication: lytic or lysogenic cycle

The study of the events that occur in an infected cell can be distinguished into three phases, named: adsorption, multiplication, liberation. For describing this cycle, is necessary to consider separately two main categories of bacteriophages: virulent and temperate.

- Virulent bacteriophages, as T7 phage, contribute to the *lytic cycle*. It begins with a process of adhesion between the basal plate, the fibrils of the phage and the lipopolysaccharide of the bacterial cell wall. The degree of specificity of this process is equal to that which it has in the interaction of an antibody with the corresponding antigen.

  The propagation of the virus depends solely by the presence of viral DNA in the host. This was confirmed by the observation that, if the permeability of the bacterial cell wall is increased with an appropriate treatment, the bacterium can be infected with DNA purified phage, with exactly the same result if it were infect by the whole virus [25].

  It became also clear that the function of the protein components of the virus is to protect the nucleic acid during its passage from one cell to another and in facilitating a specificity of penetration into a host cytoplasm.

  In particular, after the absorption of the tail of the phage, its sheath is contracted which makes penetrate the central part of the tail across the

cell wall and the cytoplasmic membrane, thus coming in contact with the cytoplasm of the host. The penetration of the phage DNA into the host, determines an almost immediate suspension of the biosynthesis of DNA, RNA and proteins: the only de novo biosynthesis of these polymers is encoded by the phage genome. After a latency period, the synthesis of DNA resumes, but it is completely limited to the production of viral DNA. Then, the multiplication starts.

After the infection, only a small amount of RNA is processed and it seems that it is intended solely phage mRNA. It transcribes the information for the synthesis of specific proteins of the virus, which are produced according to a temporal sequence. The first are enzymes that participate in the production of phage DNA, then structural proteins necessary for the maturation of new phage particles are processed. Together with phage lysozyme they participate in the eventual lysis of the host cell. In consequence of all these processes, in the cytoplasm of the host, all the constituents of the particles of the mature virus are now accumulated.

The first stage of the 'assembly' of new viruses consists in the condensation of the phage DNA into the structural elements with the shape of phage heads is induced by a special 'condensing' protein. This condensation is followed by the crystallization of the protein subunits of the head around the DNA, so as to form mature heads. Tail and the basal plate are then processed by the condensation of appropriate protein subunits and, finally, the tails and heads are combined. The final stage seems to consist in the synthesis and assembly of the fibrils of the tail. Each stage of the aggregation of a structural component is dependent on the completion of the previous stage. When the processing of new phage particles is complete, the lysis of the host cell occurs, followed by the liberation of some hundreds of phage particles that can infect other host cells.

Since the whole process of multiplication takes only ten to twenty minutes, the reproductive potential is clearly enormous and just limited by the availability of an adequate number of host cells.

• A lambda or M13 phage, belongs to the categories temperate phage. This

takes an alternative algorithm than the previous named *lysogenic cycle*. The viral DNA enters the host as described, but instead of causing cell death by lysis, it is incorporated as a component of the host genome: the 'prophage'. It then replicates as if it was a part of the normal bacterial genome and only occasionally a lytic cycle of development breaks out spontaneously. The impact of multiplication step can be increased by some inducing agents.

The presence of a prophage, as component of the bacterial genome, changes some host functionality: first, the lysogenic bacterium becomes immune from attacks by other phages, but even more important, there may be alterations of specific bacterial genotype, called lysogenic conversions, which don't presents obvious relationship with the biological cycle of the phage. For example the surface antigens of the bacterium can be modified or it can be altered the toxin production [26]. Figure 10 shows the cycles steps.

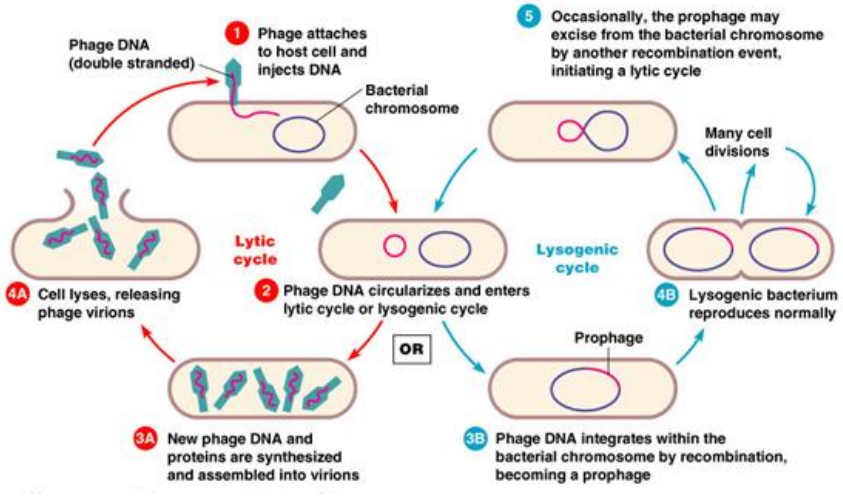


Figure 10: Lytic and lysogenic cycle copyright for a virulent and temperate bacteriophage, respectively. *Pearson education, 2004*.

3.1.3 Bacterial and phage quantification

- Bacterial enumeration. In the study of microbiology, there are numerous occasions when it is necessary to either estimate or determine the number of bacterial cells in a broth culture or liquid medium. Determination of cell numbers can be accomplished by a number of direct or indirect methods.

Standard Plate Count (Viable Counts)

A viable cell is defined as a cell which is able to divide and form a population or colony. A viable cell count is usually done by diluting the original sample, plating aliquots of the dilutions onto an appropriate culture medium, then incubating the plates under proper conditions so that colonies are formed. After incubation, the colonies are counted and, from knowledge of the dilution used, the original number of viable cells can be calculated (Fig. 11). For accurate determination of the total number of viable cells, it is critical that each colony comes from only one cell, so chains and clumps of cells must be broken apart. However, since one is never sure that all such groups have been broken apart, the total number of viable cells is usually reported as colony-forming units (CFUs) rather than cell numbers. This method of enumeration is relatively easy to perform and is much more sensitive than turbidimetric measurement. Thus, the concentration of bacteria in the original suspension is calculated using the following formula:

$$\frac{CFU}{ml} = \frac{Number\ of\ colonies\ counted\ in\ the\ plate}{Volume\ plated\ (ml) \cdot Dilution\ factor} \qquad (9)$$

A major disadvantage, however, is the time necessary for dilutions, platings and incubations, as well as the time needed for media preparation.

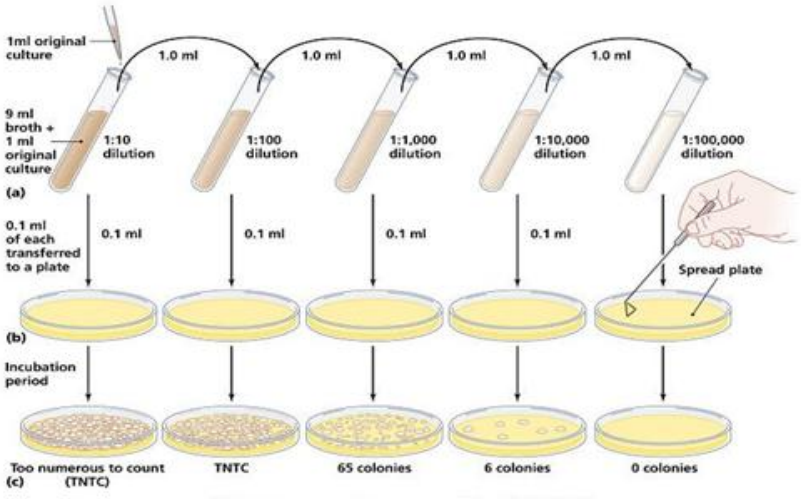


Figure 11: illustration of the protocol related to standard plate count (SPC) test. *Pearson Education, 2006*.

Turbidimetric Measurement

A quick and efficient method of estimating the number of bacteria in a liquid medium is to measure the turbidity or cloudiness of a culture and translate this measurement into cell numbers. This method of enumeration is fast and is usually preferred when a large number of cultures are to be counted.

Although measuring turbidity is much faster than the standard plate count, the measurements must be correlated initially with cell number. This is achieved by determining the turbidity of different concentrations of a given species of microorganism in a particular medium and then utilizing the standard plate count to determine the number of viable organisms per milliliter of sample. A standard curve can then be drawn, in which a specific turbidity or optical density reading is matched to a specific number of viable organisms. Subsequently, only turbidity needs to be measured.

Turbidity can be measured by an instrument such as a colorimeter or spectrophotometer (Fig. 12). These instruments contain a light source and a light detector (photocell) separated by the sample compartment. Turbid solutions such as cell cultures interfere with light passage through the sample, so that less light hits the photocell than would if the cells were not there. Turbidimetric methods can be used as long as each individual cell blocks or intercepts light. Thus, if the mass of cells becomes so large that some cells effectively shield other cells from the light, the measurement is no longer accurate.

Before turbidimetric measurements can be made, the spectrophotometer must be adjusted to 100% transmittance (0% absorbance). This is done using a sample of uninoculated medium. Percent transmittance of various dilutions of the bacterial culture is then measured and the values converted to optical density, based on the formula:

$$\text{Absorbance (O.D.)} = 2 - \log \% \text{ Transmittance} \qquad (10)$$

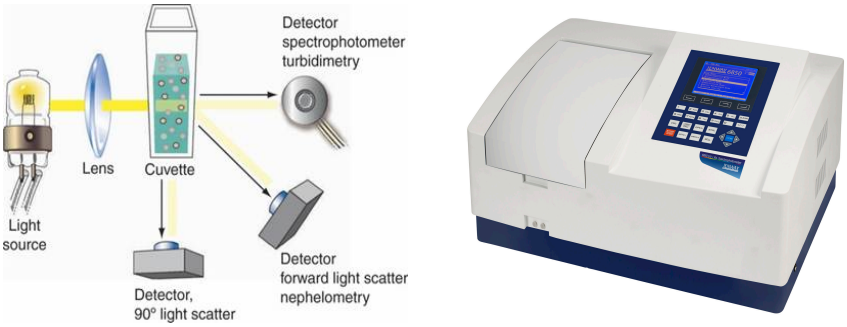


Figure 12: al optical arrangements of nephelometry and turbidimetry; **b**l a commercial spectrophotometer.

Direct Microscopic Count

Petroff-Hausser counting chambers can be used as a direct method to determine the number of bacterial cells in a culture or liquid medium. In this procedure, the number of cells in a given volume of culture liquid is counted directly in 10-20 microscope fields (Fig. 13). The average number of cells per field is calculated and the number of bacterial cells ml^{-1} of original sample can then be computed. A major advantage of direct counts is the speed at which results are obtained. However, since it is often not possible to distinguish living from dead cells, the direct microscopic count method is not very useful for determining the number of viable cells in a culture.

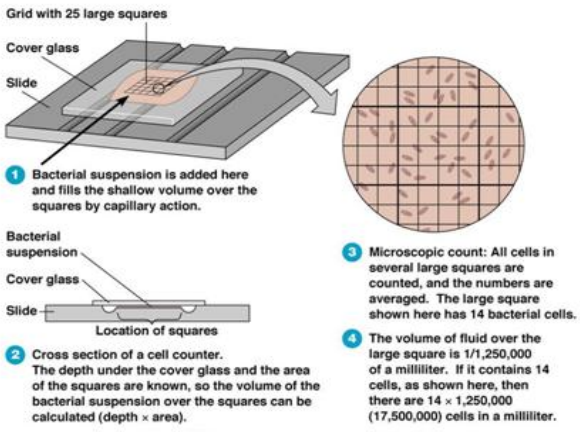


Figure 13: illustration of the protocol related to direct microscopic count on petroff hausser counting chamber. *Pearson Education, 2007*.

Enumeration of phage particles. The plaque assay is a virological assay employed to count and measure the infectivity level of the bacteriophages. The basis of plaque assay is to measure the ability of a single infectious virus to form a “plaque” on a concurrent monolayer culture cells. Aliquots of diluted bacteriophage are mixed with host bacterial cells in several milliliters of soft agar, which are then spread onto agar plates containing media. The use of soft agar allows the phage to easily diffuse through the medium giving more consistent plaque formation. It also eliminates the problem of uneven absorption of the bacterial-phage solution into the hard agar that often caused uneven plaque formation on the plate [27].The bacteriophage adsorb onto the host bacterial cells, infect and lyse the cells, and then begin the process anew with other bacterial cells in the vicinity. After 6 – 24 hours, zones of clearing, plaques, are observable within the lawn of bacterial growth on the plate (Fig. 14).

Thus, the concentration of bacteriophage in the original suspension is calculated using the following formula:

$$\frac{PFU}{ml} = \frac{Number\ of\ plaques\ counted\ in\ the\ plate}{Volume\ plated\ (ml) \cdot Dilution\ factor} \qquad (11)$$

Plaque characteristics are related to the type of bacteriophage as well as other physical and chemical characteristics of the system in which the bacteriophage are grown [28].

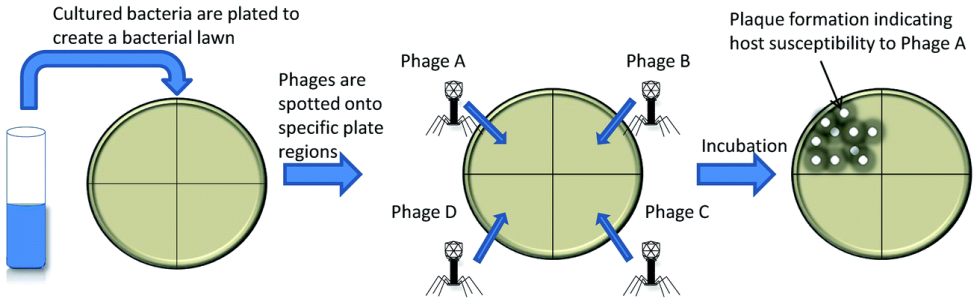


Figure 14: illustration of the protocol related to plaque assay.

3.2 General principle of bacterial continuous culture

Prior to its development, the bacterial culture was carried out in closed systems, a lot or batch, in which the microorganism was inoculated and cultured. The growth continues until the soil is not more suitable, due to the depletion of nutrients or to the accumulation of toxic products from bacterial growth itself. The culture, thus, enters in a stationary phase, where no evident growth occurs, and after a variable period of time extinction occurs.

Under conditions of continuous culture rather, the feeding of nutrients is continuous and controlled. Then, the culture is maintained in the same growth conditions even for long periods of time. All continuous flow systems consist essentially of **a** reactor into which reactants flow at a steady or dynamic rate and from where products emerge.
The factors governing their operation are:

- the way in which material streams through the volume of reactor. This depends by the design of the configuration chosen.
- the kinetics of the reaction taking place.

3.2.1 Fermenter

A fermenter is a culture vessel where microorganisms can be grown under suitable conditions. Sterile growth medium is fed into the culture vessel at a flow-rate (***f***) and culture emerges from it at the same rate. A device keeps the volume of culture in the vessel *(**v**)* constant. The contents of the vessel are sufficiently well stirred to approximate to the ideal of complete mixing, so that the entering growth medium is instantaneously and uniformly dispersed throughout the vessel.
Residence-times in such a culture vessel will be determined not by the absolute values of the flow-rate and culture volume but by their ratio which we call the dilution rate, ***D*** [h^{-1}]***,*** defined as ***D=f/v,*** i.e. the number of complete volume-changes/hr. The mean residence-time of a particle in the culture vessel is thus equal to **1 / *D.***
For understanding which are the time characteristics of a continuous reactor, we assume that the bacteria in the culture vessel are not

growing or dividing. With complete mixing, every organism in the vessel has an equal probability of leaving it within a given time. It can easily be shown to follow that the fraction of the total organisms in the vessel having a *residence-time* at is e^{-Dt}. The *wash-out rate*, i.e. the rate at which organisms initially present in the vessel would be washed out if growth ceased but flow continued is therefore :

$$\frac{-dx}{dt} = D \cdot x \qquad (12)$$

where x is the concentration of organisms in the vessel. The distribution of *residence times* and the *wash-out rate* in a completely-mixed continuous culture vessel of this sort can thus be adequately described in fairly simple terms. Therefore, if the kinetics of the culture is known, this tool can be used for the technique of continuous culture.
Monod (1942) first showed that there is a simple relationship between the specific growth rate and the concentration of an essential growth substrate, (***μ***) being proportional to the substrate concentration when this is low but reaching a limiting saturation value at high substrate concentrations according to the equation

$$\mu = \mu_m \cdot \left(\frac{s}{s+K}\right) \qquad (13)$$

where *s* is the substrate concentration, **,** μ_m is the growth rate constant (i.e. the maximum μ value of at saturation levels of substrate) and *K* is a saturation constant numerically equal to the substrate concentration at which $\mu = 0.5 \cdot \mu_m$. It follows from equation (13) that exponential growth can occur at specific growth rates having any value between zero and μ_m, provided the substrate concentration can be held constant at the appropriate valuea fact of major importance in continuous culture [29]**.**

- Chemostats are continuous cultures grown in fermenters with control systems, which are widely used for research in microbial physiology [30]. Parameters, like temperature, pH and concentration of dissolved gases in the growth medium, can be monitored and controlled. Fresh medium is supplied to the culture at a constant flow rate ***F***, and removed from the

fermenter at the same rate, thus maintaining a constant culture volume ***V***. As we already said, at steady state, the growth rate of the culture equals to the dilution rate, ***D = F / V***, and the biomass concentration and other free parameters stabilize at levels depending on the dynamics of the fermentation. Hence, chemostats allow continuous exponential growth to occur for many generations under constant physicochemical conditions. Crucially, chemostat culture allows the experimenter to control the growth rate (flux at any value below a maximum value termed the maximum specific growth rate (D_{max}). This contrasts with batch cultures, where biomass concentration and the environmental conditions (in terms of pH, nutrient concentration etc. change significantly during the (limited -me course of the fermentation, and the experimenter has no control over growth rate flux.

The factor that determines the growth rate of a cell population in a chemostat is dilution rate, *i.e.* the rate of supply of the limiting nutrient. In a chemostat operating at a low dilution rate $D<<D_{max}$, the limiting nutrient is present at very low concentrations at the steady state. Therefore, most of the nutrient is converted into cells and the biomass concentration is high (close to the cell concentration of an equivalent batch culture at late exponential phase). As the dilution rate is increased, the availability of the limiting nutrient increases; however, the rate of removal of cells from the growth vessel is also higher and so the biomass concentration falls. At values of $D\approx D_{max}$, nutrient limitation entirely disappears as there are too few cells in the culture to utilize the available nutrients. Those cells remaining in the culture vessel grow at their maximum growth rate since even the limiting nutrient is present in excess. Finally, at $D>D_{max}$, a steady state can no longer be maintained and the number of cells in the fermentors starts to fall since the rate at which new cells are produced is insufficient to over

come dilution by the addition of fresh medium, which leads to the washout of the cells from the growth vessel.

- Turbidostats, are continuous cultures reactor in which the cells are under growth rate controlled condition [31]. A turbidostat employs a positive feedback control system that senses, and responds to, the biomass concentration in the fermentor. If the culture is growing faster than the rate imposed by the current dilution rate, then the biomass concentration will rise as a result of the positive difference between the biomass production rate and the rate of removal of cells from the culture vessel. In such circumstances, a control loop is activated that increases the dilution rate by increasing the speed at which a pump introduces fresh medium into the culture. This continues until the biomass concentration is decreased to some set point (which, in our experiments, represents the biomass concentration of amid exponential phase batch culture growing in the same medium under the same conditions. On the other hand, if the culture is growing slower than the current dilution rate, the biomass concentration will fall to below the set point, and the control loop acts to decrease the rate at which fresh medium is pumped into the growth vessel. Hence the turbidostat equilibrates at a dilution rate equal to the actual growth rate of the culture and the cell concentration stays constant. As our set point is equivalent to the biomass concentration of a mid exponential phase batch culture, the turbidostat equilibrates at the maximum specific growth rate of the yeast strain used.

Figures 15 shows the schematic configuration for the bioreactor presented.

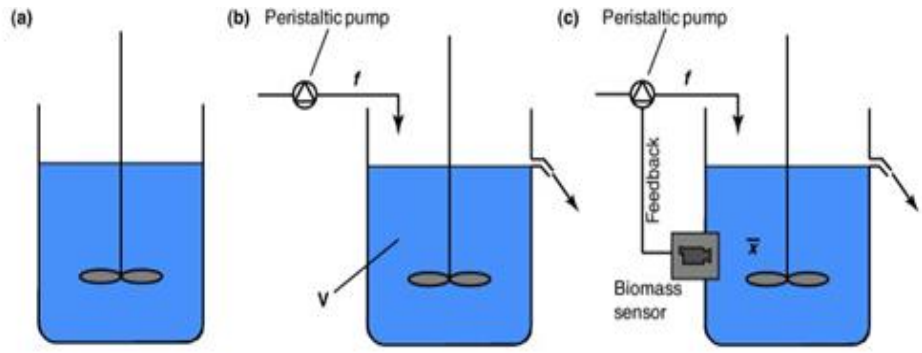


Figure 15: al batch culture; **bl** bioreactor in configuration chemostat with costant D=F/V; **cl** bioreactor in configuration turbidostat with D=f/V controlled by biomass (OD) sensor.

3.3 Phage-assisted continuous evolution (PACE)

As the products of an evolution are closely linked to the number of generations that characterize the process, PACE, is an innovative technique that combines the continues viral cultivation described by Husimi [32] and the protein production in E. coli.

To maximize the round, the protocol uses the bacteriophage, the fastest evolutionary replicator known in nature. Its effectiveness, which can reach a dozen cycles per day, is thus assessed by quantifying the protein or nucleic acids that constantly evolving take desired functions in vivo. [33], [34].

The PACE is performed linking the phage's ability to reproduce to the desired molecular activity, by moving a gene required for phage infection from the phage genome into the host cell and replacing it with the gene(s) to evolve. The host cell has been engineered to produce the missing phage protein in proportion to the desired activity. Thus, in a continuous culture apparatus where the amount of evolutionary substrate, i.e. bacterial density, is constant, the free phages infecting compete to optimize the function of the gene(s) to be evolved in order to maximize the amount of phage protein produced, and thus their own fitness. Otherwise, they will be rinsed periodically from the volume in which they are set with the excess of bacterial culture, causing the extinction or domination of a phage effectively functionalized. To perform PACE experiments, the basic setup consists of two bioreactors. The first is a chemostat or a turbidostat, where uninfected (i.e. with bacteriophage) cells are

continuously cultivated. The cells are then pumped into a second bioreactor, a lagoon, where they can come into contact with the phage, and become infected. The dilution rate of this vessel is kept sufficiently high, so that cells that enter will, on average, be pumped out of the lagoon before they have a chance to divide. However, there is still enough time for the bacteriophage population to reproduce, as the phage reproductive cycle is much faster than that of the cells. This ensures that only the bacteriophage is able to undergo evolution, so by applying specific evolutionary pressures, it is possible to use this system to evolve specific biomolecules.

The only directed evolution that has been carried out using this method, to date, is the work performed by Liu, *et al* (2011). They managed to link the evolution of RNA polymerases to the production of protein III. This is a capsid protein on the M13 bacteriophage that infects E. coli cells, required for phage infection, as it is responsible for F. pilus binding and it is also necessary for the detachment of new phage particles from the cell [35]. By removing gene III, which codes for protein III, from the M13 genome and placing it in an accessory plasmid within the E. coli cells, phage propagation is dependent on its ability to initiate protein III production from the accessory plasmid AP. Only phage vectors able to induce sufficient pIII production from the AP will propagate and persist in the lagoon (Fig. 16). Moreover, because pIII expression level determines the rate of infectious phage production, phage encoding genes that result in a higher level of pIII production, “selection phage” (SP), will infect more host cells than phage encoding less active genes. Indeed, Phage lacking pIII are ~10^8-fold less infectious than wild-type phage.

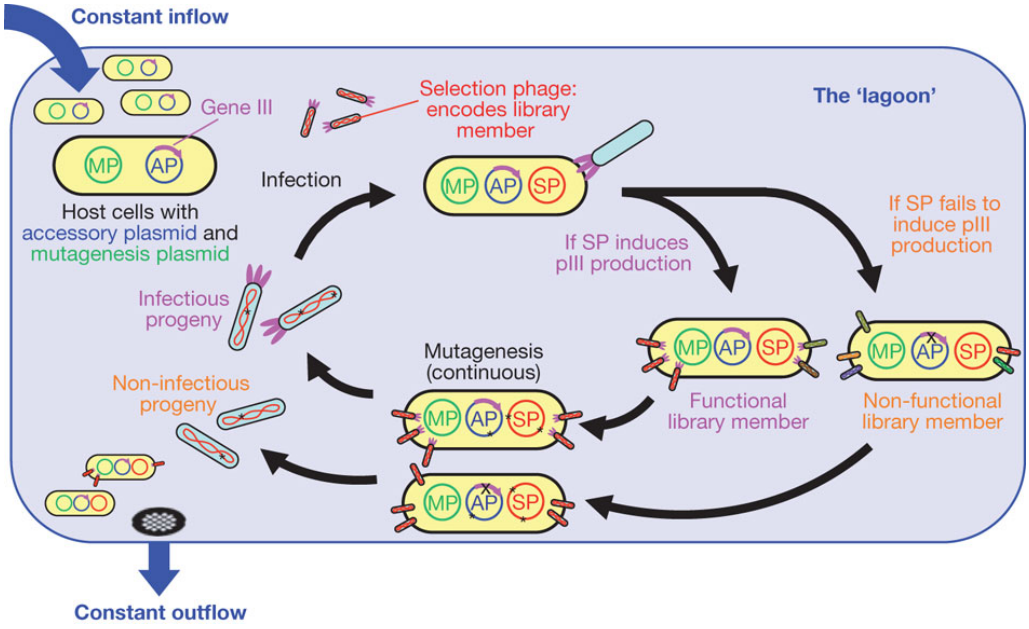


Figure 16: *overview of the PACE system. PACE in a single lagoon.* Host cells continuously flow through a lagoon, where they are infected with selection phage (SP) encoding library members. Functional library members induce production of pIII from the accessory plasmid (AP) and release progeny capable of infecting new host cells, whereas non-functional library members do not. Increased mutagenesis is triggered through induction of the mutagenesis plasmid (MP). Host cells flow out of the lagoon on average faster than they can replicate, confining the accumulation of mutations to replicating phage. *Liu, et al; Fig.1, Nature, (2011).*

So PACE applies optimal evolutionary pressure when pIII levels are above the minimal threshold required to prevent phage washout, but below the amount needed to maximize infectious phage production. This window can be shifted by varying the copy number of the AP, or by altering the ribosome-binding site (RBS) sequence of gene III to modulate the efficiency with which gene III is transcribed or translated. Also, any mutation of host cells has a minimal impact on the outcome of the selection over many rounds of phage replication because the lagoon dilution rate is so fast that only the evolving selection phage population can replicate. A second plasmid called "Mutagenesis Plasmid" (MP) has been included in the cell hosts. This arabinose- inducible mutagenesis plasmid promotes the evolution elevating the error rate during DNA replication in particular suppressing proofreading and enhancing error-prone lesion bypass.

3.4 Evoprog Consortium and Jaramillo's Lab research

3.4.1 A technique for a lysogenic phagemid evolution into F⁺ E.coli cells

As previously introduced, the original idea for PACE was proposed some time ago, but research using the system as a method for directed evolution has only been carried out very recently. This context generates the Evoprog Consortium, whose goal is to build a general purpose evolutionary machine that increases the speed and efficiency of the PACE technique, by utilizing miniaturized and multiple bioreactors interconnected and automated. It also uses ad hoc designed biological parts, such as bacteria cells, plasmids and phagemid instead of phages as the vector for transferring the evolving genes between host cells. By doing so, it should be possible to observe evolution over even shorter timescales than the previously experiment, due to the creation and maintaining of faster reproductive cycle conditions for phagemid compared with filamentous, or lysogenic, M13 bacteriophage (Fig. 17).

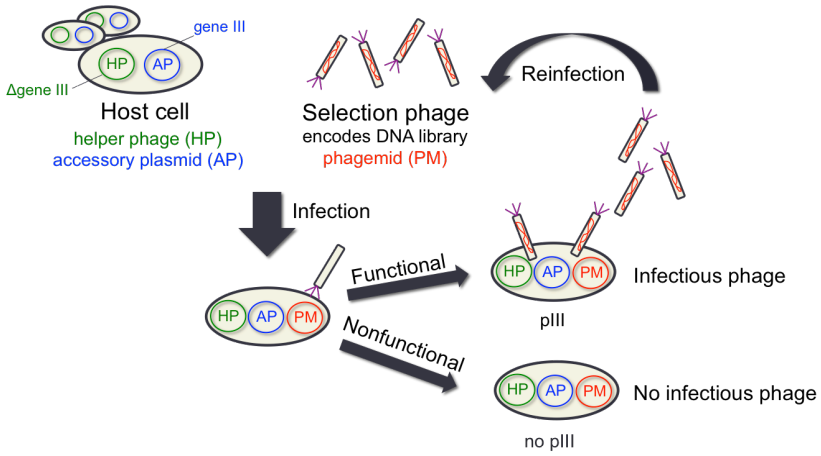


Figure 17: *fundamental logic behind the (lysogenic) phagemid evolution system into F⁺ E.coli cells. E. coli* cells containing the M13 helper phage (HP) system are infected with selection phages encoding a target gene (which is mutated in the host cell) or a combinatorial DNA library. After infection, only a protein with desired characteristics leads to viral gene III expression (pIII) and thus infectious phage production.
Courtesy of Dr. M. Isalan, Imperial College (UK), Evoprog partner.

Because of the phagemid, an helper phage plasmid (HP) into the F⁺ E.Coli host cells provides all phage components except the essential gene III so that, phagemid contains just the gene of interest (lambda cI) under a constitutive promoter and an M13 packaging signal. In this way, the dimension of the

vector, rather than the ultimate bacteriophage, could be massively reduced optimizing the speed of evolution process. Three different accessory plasmids were constructed. Helper phage, phagemid and accessory plasmids were equipped with different antibiotic resistances and origin of replications to ensure coexistence in the host cell.

In the next chapter an experiment about the relation between free phagemid and dilution rate of the bioreactor, will be presented.

	Relevant info	**Reference/source**
Bacterial strain		
E. coli F^+	*E. coli TG1*/$Strep^R$	Victor de Lorenzo's Lab, Centro Nacional de Biotecnología [ESP] (*Evoprog consortium partner*)
Phagemid		
M13 phagemid	pLitmus:: **Δ**Gene3/Amp^R	M. Isalan, Imperial College [UK] (*Evoprog consortium partner*)
Plasmids		
Helper Phage (HP)	M13 K07ΔpIII/Kan^R	Lab collection
Accessory Plasmid (AP)	pJPC12::Gene3/$Chlor^R$	M. Isalan, Imperial College [UK] (*Evoprog consortium partner*)

Table 1: Strains, phage and plasmids used for conducting lysogenic experiment.

3.4.2 Engineering T7 lytic phage towards a bacteriophage therapy

The current rise of antimicrobial-resistant bacteria has led to increased research interest in bacteriophage therapy. While phage therapy offers the advantage of evolvable antimicrobials, there are still issues created by the emergence of bacteriophage-insensitive mutants (BIMs). Jaramillo's Lab focuses on investigating and describing the biological kinetics between host cells and bacteriophage T7. Here two projects, where the bioreactor object of this thesis has been employed, are described.

- *Relations between lytic T7 bacteriophage concentration and F⁻ E. coli cells*

Aim of this study, is the modeling and the quantification of the kinetic of a bacterial culture when infected with different concentration of viral particles. To do this, several tests, using batch culture, have demonstrated the existence of a mathematical relation. Then, a software for phage quantification and kinetic model interference have been attained [36].

In the next chapter an experiment in continuous viral culture will be presented for describing the relation between free bacteriophage and bioreactor dilution rate.

- *In Vivo recombineering of bacteriophage T7 imparts Host-Independence F⁻ E. coli cells*

Thanks to these preliminary studies, it is possible to understand the restrictions of a bacteriophage therapy rather than an antibiotic treatment against bacterial disease. Indeed, the emergence of bacteriophage-insensitive mutants (BIMs) can affect the efficiency of the phage therapy. A likely pathway towards the emergence of BIMs may be via loss-of-function mutations in genes that are dispensable for bacterial growth but essential for phage propagation. As described by Qimron et al. (2006)[37] E. coli K12 has been screened for genes essential for lytic T7 bacteriophage propagation but nonessential for *E. coli* growth. The genes identified were *cmk* (Cytidine Monophosphate (CMP) kinase), *trxA* (Thioredoxin A) and nine genes involved in biosynthesis of the lipopolysaccharide (LPS) receptor, to which T7 binds to infect the host.

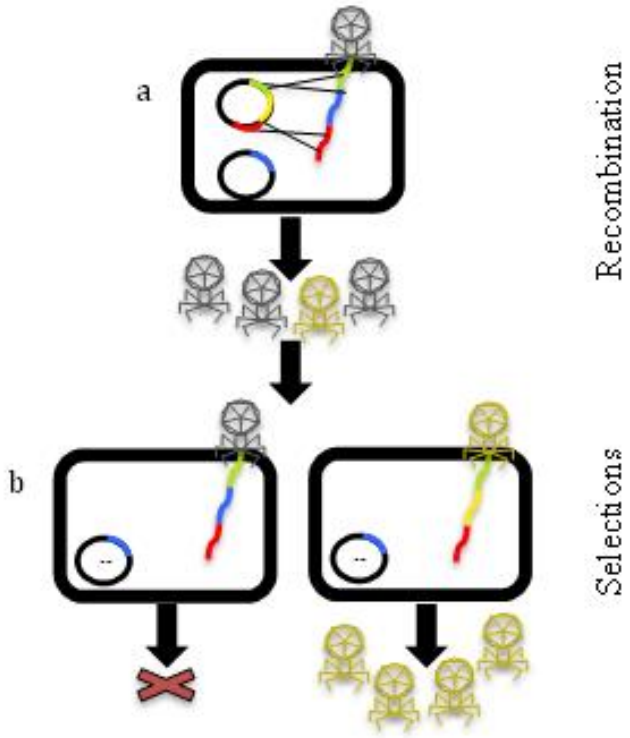


Figure 18: *fundamental logic behind an in vivo recombineering of lytic T7 bacteriophage to imparts host-independence F- E.coli cells*. Schematic of the procedure for production of T7-ReRd2Rb containing *Escherichia coli* genes *cmk* and *trxA*. **al** T7-ReRd2Rb (grey) were grown in *E. coli* expressing T7 gene 5 (blue) in trans and a plasmid with regions homologous to 100 bases either side of gene 5 in the T7 genome (green and red) and a region with *E. coli* genes *cmk* and *trxA* in-between (yellow). Homologous recombination introduced the two genes to the T7 genome. The progeny comprised a minority of recombinant phage (gold) and a majority of unedited phage (grey). **bl** The progeny were grown in *E. coli* Δ*cmk*/Δ*trxA* expressing gene 5 in trans, which only allows growth of recombinant phage, thus selecting for recombinant phage on a phenotypic basis.
p1 = pSB3T5::*cmk*/*trxA*; **p2** = pET24::Gene5/Kan^{R}; **p3** = pET24::Gene5/Amp^{R}
Courtesy of M. Tridgett, MBITP project, 2014/15

For this aim, a variant of T7 bacteriophage, has been engineered by homologus recombination for inserting all these three factors: *cmk, trxA and LPS* in its genome followed by plaque assays against doubly knocked-out for *cmk* and *trxA E. coli* to select for recombinant phage. To edit the phage genome, T7 was grown in a strain of *E. coli* that contained a plasmid with sequences homologous to regions in the phage genome, facilitating homologous recombination [38]. A second strain (targeting strain), containing

a CRISPR-Cas[9] system targeted to the unedited T7 locus, was then used to select against unedited phage genomes. Gene 1.7 was deleted in the study by Kiro et al. (2014), whereas homologous recombination was used here to insert *cmk* and *trxA*, and selection was performed on a phenotypic basis (Fig. 18).

The next chapter describes an experiment to demonstrate the homologous recombination of T7 phage in continuous culture.

	Relevant genotype	**Reference/source**
Bacterial strain		
E. coli F-	*E. coli* MG1655::Gene5/Kan^R	Lab collection
Recombination strain	*E. coli* MG1655::Gene5/Kan^R pSB3T5::*cmk*/*trxA*	M. Tridgett's study, *Univ. of Warwick* [UK]
Selection strain	*E. coli* Δ*cmk* Δ*trxA* MG16555::Gene5/Amp^R	
Phage		
T7-ReRd2Rb	Variant in genes 11, 12 and 17	Qimron (2006)
RwtT7	wtT7::*cmk*/*trxA* ΔGene5	M. Tridgett's study, *Univ. of Warwick* [UK]
T7-HI	T7-ReRd2Rb::*cmk*/*trxA* ΔGene5	
T7-G5	wtT7ΔGene5	J. Hassal's study *Univ. of Warwick* [UK]
Plasmids		
Recombination Plasmid (RP)	pSB3T5::*cmk*/*trxA*	M. Tridgett's study, *Univ. of Warwick* [UK]
Accessory Plasmid (AP)	pet24_gp5/ Kan^R	J. Hassal's study *Univ. of Warwick* [UK]

Table 2: Strains, phage and plasmids used for conducting lysogenic experiment.

[9] CRISPR-Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) system, a bacterial immunity system that can be reprogrammed to cleave a desired nucleic acid sequence.

Chapter 4

4 Prototype and results

Despite the development of instruments such the ones described in chapter II, commonly the experiments of directed evolution are linked to the experimenters skills, that laboriously creating new generations of candidate molecules, perform a selection by hand, extract the DNA of the best performers, and repeat the cycle.
The effectiveness of these studies can be improved from the technological point of view, maximizing the total number of rounds that take place in the evolutionary process by identifying what are the features that accelerate it, as dilutions and temperatures. The device developed throughout this thesis, must then be able to select and maintain the conditions of the system that will provide the host cells, in order to support the maximum rate of evolution of the viral population, avoiding its extinction.

In this chapter the studies of the kinetics of biomolecules that have led to the creation of a prototype modular bioreactor are described. They allowed to evaluate, in two different experiments, the presence of the population of lytic or lysogenic phages. In addition, a first approach to the interconnection of independent bioreactors is illustrated in order to obtain an example of in vivo recombinant phage.

4.1 A mathematical model for describing a lysogenic phagemid ACE system

A mathematical model is presented in a paper by Husimi to describe the dynamics of the PACE system, with regards to the number density of free M13 lysogenic bacteriophage, and E. coli cells, in their various infection states into the volume of a cellstat. This has been adapted to describe the system used for this work, where M13 phagemid is used in conjunction with F^+ TG1 Helper Phage (HP) cells.
Thus, under the adapted model for a turbidostat where a continuous bacterial

culture is kept to an exponential phase, the cells and phagemids into the cellstat, undergo the reaction kinetics shown in (Fig. 19). The infection rate of uninfected cells with free phagemid is denoted by k_1, k_2 is the rate at which infected cells become infectious (i.e. there is a latent period), and k_3 is the secretion rate of fresh phagemid from cells in state *Y*. Additionally, k_g is the growth rate of the cells.

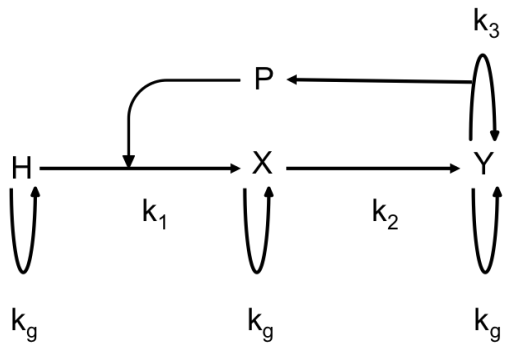


Figure 19: '*Reaction kinetics' diagram*, describing the interaction of cells and free phagemid within the cellstat. Short bars crossing an arrow indicate the multiplicity of the arrow.

The original model ignores the possibility of cells becoming infected with multiple phagemids because is known that infectous E. coli cells with M13 phage produce an immunological response, which inhibits further infections. However, no such response is believed to occur after infection of TG1 HP cells with M13 phagemid, so multiple infection can occour. Additionally, Husimi's model contains a 'Z' state for the host cells, which is a post- infectious state where cells no longer secrete new phage particles. For the use of the model in a cellstat volume, this is ignored, as we have no simple way of differentiating cells in this state from others, or measuring the rate at which cells enter this state because of high enough dilution rates.

Tge equations describing the system from Fig. 19, are

$$\frac{dh}{dt} = D_c \cdot (h_0 - h) - k_1 \cdot h \cdot p + k_g \cdot h,$$

$$\frac{dp}{dt} = -D_c \cdot p - k_1 \cdot h \cdot p + k_3 \cdot y,$$

$$\frac{dx}{dt} = -D_c \cdot x - k_2 \cdot x + k_1 \cdot h \cdot p + k_g \cdot x,$$

$$\frac{dy}{dt} = -D_c \cdot y - k_2 \cdot x + k_1 \cdot h \cdot p + k_g \cdot y \qquad (14)$$

Here,

h, density of uninfected cells in the cellstat
h0, density of uninfected cells from the chemostat/turbidostat
x, density of infected cells in their latent period (i.e. before they begin secreting phages)
y density of infected cells that secrete daughter phagemid
p free phagemid density
the rate constants are as defined in (fig. 19).
Additionally, $D_{c=f/v}$, is the dilution rate of the cellstat.

These equations are only valid for describing cell dynamics in the cellstat when it is connected to a turbidostat at a low OD, as no growth limiting factors are included. The equations can be modified to describe a chemostat, by substituting the growth rate for a reduced value fk_g, where $0 < f < 1$. For a given dilution rate in the chemostat, f is easily determined, as when the chemostat is operating in steady state, fk_g will equal $D_{chemostat}$.

From these equations, the steady state solutions for the system are found to be

$$\hat{h} = \frac{Dc}{[k1\ k2\ k3/(Dc+k2-kg)(Dc-kg)]-k1},$$

$$p = h_0 \frac{k2\ k3}{(Dc+k2-kg)(Dc-kg)} - 1 - (Dc-kg)/k1,$$

$$x = \frac{k1\ \hat{h}\ p}{Dc+k2-kg},$$

$$y = \frac{k2\ x}{kg-Dc} \qquad (15)$$

It can be seen that the steady state values of *h* and *p* depend on the dilution rate in the cellstat, whilst *p* also depends on the density of cells arriving from the turbidostat. It is perhaps surprising that the steady state value of *h* does not depend on h_0, and only on the dilution rate. One way to think about this is that h_0 is itself determined by the dilution rate in the first bioreactor. Also, it was previously thought that E. coli cells cultured in a chemostat would not be suitable for PACE experiments, as the F-pili, which are required for infection by M13 phage, do not form for cells grown outside of the exponential growth phase [39]. However, recent experiments performed by Liu, *et al* (2014), show

that this is not the case, and cells grown in a chemostat can be suitable for use in PACE experiments.

These two clarifications confirm and therefore justify the use of a simple chemostat for cell cultivation. The dimensioning of the cellstat is only possible if you know within which values of dilutions can maintain a lysogenic phage population without compromising the cellular density in the first volume, knowing the doubling time of cells infected or without phagemid.

The next studies in the bioreactor are then routed to determine those values of dilutions of volume per unit of time in cellstat in which the above conditions are met.

4.2 A preliminary mathematical model for describing a lytic bacteriophage

Regarding only the modeling of a volume of cellstat, where lytic T7 bacteriophages are fed with cells, we will consider here a model described by the paper "Understanding bacteriophage therapy as a patented density dependent kinetic process" (Payne & Jansen, 2000).

However a parallel project of this thesis is throughly studing the matematical aspect of the lytic bacteriophage kinetic (N. Papili Gao, 2015).

$$\frac{dx}{dt} = -a \cdot x - b \cdot v \cdot x - H(t) \cdot x$$

$$\frac{dy}{dt} = -a \cdot y - b \cdot v \cdot x - k \cdot y - H(t) \cdot y$$

$$\frac{dv}{dt} = -k \cdot L \cdot y - b \cdot v \cdot x - m \cdot v - h(t) \cdot v \qquad (16)$$

Here,

x(t) represents the concentration of uninfected bacteria
y(t) the lytic bacteria
v(t) the free phage
H(t) is the host response against the bacteria
h(t) is the host response against the phage
a is the replication coefficient of the bacteria
b is the transmission coefficient
k is the lysis rate coefficient
L is the burst size
m is the decay rate of free phage.

From a first analysis it is clear that for the realization of the bioreactor the basic parameters, in the case of lytic phage, are related to the kinetics of host cells: density and doubling time. Moreover, because the phage is lytic, it must considered the time to burst at different concentrations of phage.
It justifies the next studies relating to the maintenance in the bioreactor of the population of phage, in relatively constant concentrations over a long period of experimentation without any effect of wash out or total domination of number of phage on host cells. Also in this case, we are thus interested on the concentration of phage at different dilution rate and temperature into the volume of cellstat.

4.3 Kinetic characterization of the host cell

In order to build a prototype that can maintain phage population over time ensuring optimal conditions of development, it is necessary to determine experimentally what are the values of dilution within the volume of cellstat or phagereactor that do not cause the wash out of the lytic or lysogenic phage population. For this reason, we will focus on the growth curves of infected or uninfected cells, identifying those values of volume per unit time which, however, do not compromise the fundamental condition of constant density of susceptible host cells for free cloning vectors in the volume of cellstat.

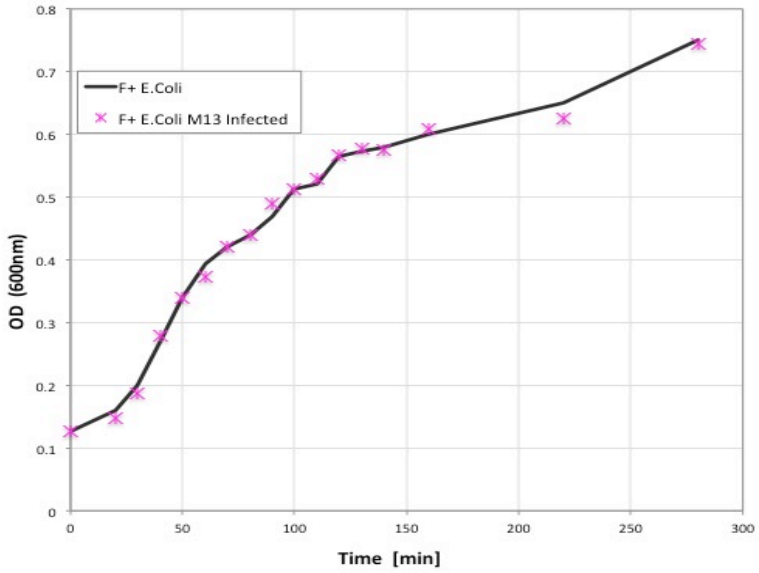
4.3.1 Growth curve of lysogenic phagemid infected and uninfected F^+ E. coli cells

The plot of OD against time for the growth rate experiment is shown in Graph 1. It can be seen that at around 250min, the OD starts to level off, as the cells enter the stationary growth phase. The doubling rate of 1.77h^{-1}, and a doubling time of approximately 34 minutes have been calculated.
Previously, a growth rate of 1.2 ± 0.1$h^{-1}$was measured within our group for uninfected TG1 HP cells, which also corresponds to a doubling time of approximately 34 minutes. It therefore appears to be the case that the growth rate of TG1 HP cells does not detectably change after becoming infected with

M13 phagemid. As the genome of phage is very short, it is reasonable to consider that this does not introduce any effect on the replication time of the cells.

From the graph, it can be understand that for the lysogenic phage the time of growth is related to the minimum dilution of the cellstat that does not allow, on average, the duplication of bacteria in the same volume.

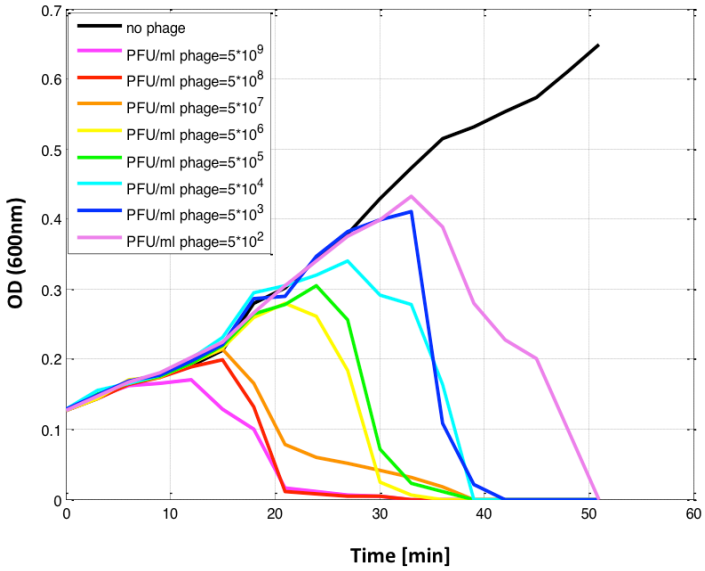
Thus, we could confirm that the minimum dilution in cellstat must be greater than the Dc=1,76h^{-1}.



Graph 1: shows the plot of OD against time for all the data obtained. The growth rate have been estimated plotting the ln(OD) against time up to the data point of 100min (not shown). Positive and negative controls not shown.

4.3.2 Growth curve of lytic bacteriophage infected and uninfected F⁻ E. coli cells

Regarding the study of the growth of bacteria infected by lytic bacteriophage it must be underlined that unlike the lysogenic case, here is introduced the phenomenon of burst of cells in intervals time that depend on the concentration, of bacteriophage expressed in MOI or PFU. For this reason the curves of bacteria infected with different concentrations of phage are shown in the following graph 2.



Graph 2: OD against time for concentration of bacteria cells infected by different dilution of phages. Negative control: no phage; positive control not shown.

From the graph it can be identified a window by which determine the rates of dilution of the cellstat. Indeed, with too low dilutions, the population of phage could have an effect totally dominant within the volume cellstat such as to cause burst of all the cells in a very short time, despite the rate of dilution. We thus consider the time value for Dc=3 h^{-1} equal to 20 min for a initial $5x10^9$ PFU/ml.

If an excessive dilution is setup, not enough time would give to the phage for infecting and lysing a roughly constant amount of cells. Then, causing the phage extinction in the volume of cellstat.

To generalize the applicability of the prototype, we consider as done in the case previous lysogenic study, a minimum dilution as a limit value bigger or equal to Dc=1,76h^{-1}.

4.4 The prototype in chemostat and cellstat configuration: a basic machine for conducting ACE experiment

Doubling and burst times are now known, we can then assemble a preliminary prototype of the bioreactor.

As already stated, we chose to cultivate E. coli cells in a chemostat, in which

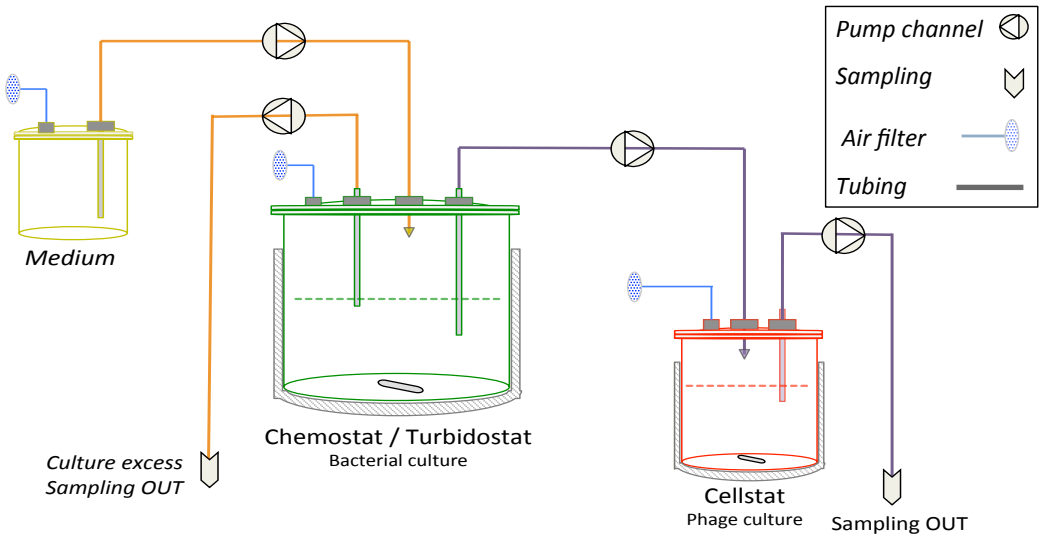
the incoming volume correspond to the outgoing, connected to a cellstat. Both are appropriately sized, taking into consideration also a daily reasonable consumptions of medium growth.

The vessels, made by common glassware lab equipment, have been interconnected using standardized tubing and fittings. In the chemostat a constant volume per hour is introduced by a peristaltic pump in order to reduce the fluctuations of bacterial density. Moreover, although we use a smaller volume for cellstat, thus an higher rate of dilution per hour is setup, the flow of the chemostat and cellstat are different. A further output has been added in order to remove the excess culture. The cellstat has instead only one input and output.

To reduce the risk of external contamination and increase the easiness of assembly, all the connections between tubings and interface with the bottles have been sealed and jointed. In particular, because the culture would have limited oxygenation, a ventilation system with pre-filtered and sterilized air, has been implemented. The air, before streaming in contact with any other solution is pumped through a 0.2um filter and sterilized with NaOH in a volume where it is temporary stored. This also allows maintaining inside of the medium reservoir a positive pressure which prevents the entrance of undesired contaminations.

To ensure a proper blending and temperature of the cultures in the vessels of chemostat or cellstat, a 3D printed multi position water bath has been designed and built. In it, can be installed three chemostat (s) and cellstat (s) independently mixed with magnetic stirrer bars and maintained, if required, at two different operating temperatures between 16 and 38°C (Appendix A).

In the scheme 1 and fig. 20 are indicated the volumes and the flow dilution in the basic configuration, type PACE, for the next experiments with M13 phagemid and bacteriophage T7.



Scheme 1: PACE, directed evolution experimental set up scheme. This configuration is currently exploited for PACE and viral continuous applications. Culture volume and flow rate are related to the kinetic biological characteristic of bacterial and viral population, for assuring the continuous culture of both, without any washout.

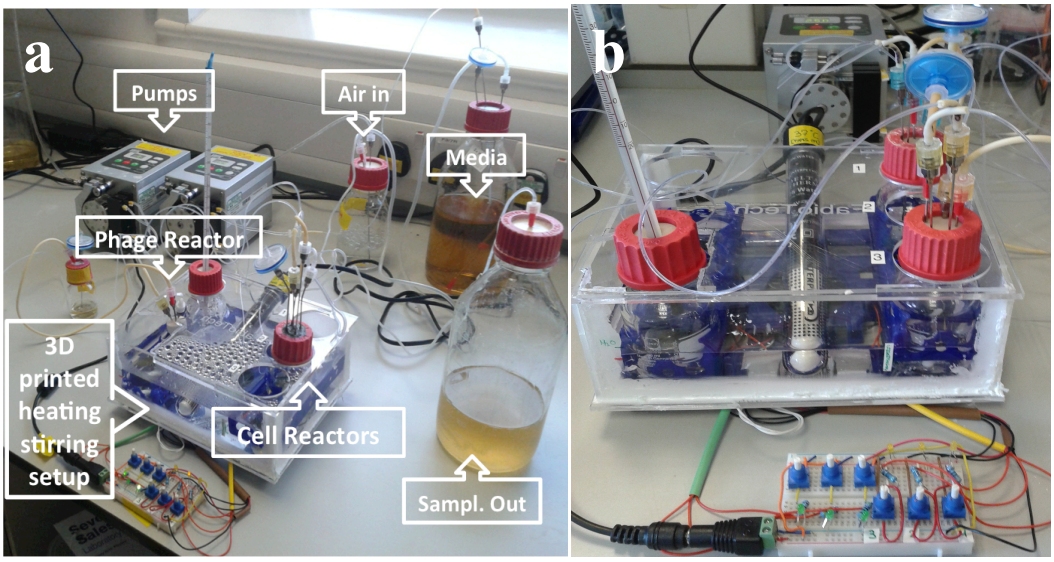
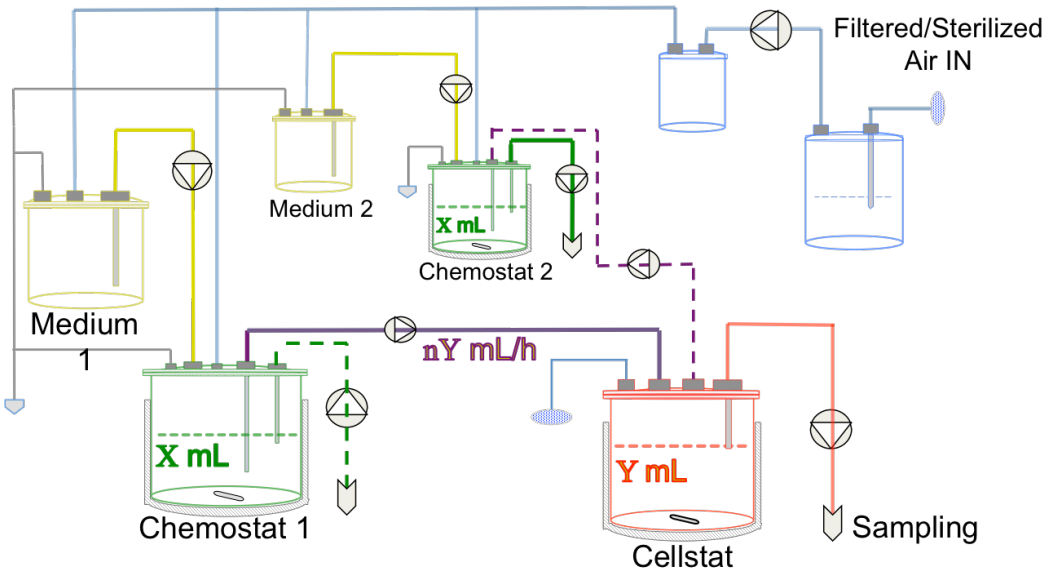


Figure 20: al overview of the prototype in chemostat and cellstat configuration; **b**l 3D printed stirring and mixing multi-spot water bath.

4.4.1 The prototype in switching configuration: a machine for a selective feed periodically switched into common cellstat

Regarding the realization of the experiment 4.7, we have modified the previous prototype such as to allow the selective use between two different bacterial cultures. Indeed, to obtain a recombination of the phage T7 in

continuous cultures, it is necessary to introduce into cellstat first one and then the other in order to vary the ecosystem that supports the phage population. To do so, we have added to the cellstat a further input and another chemostat. The logic scheme of this configuration is shown below.



Scheme 2: logic scheme of the prototype in switching configuration.

4.5 Relation between free M13 lysogenic phagemid end dilution rate

As already stated in the previous paragraphs, the aim of this prototype is to keep the population of phage, limiting the oscillations of concentration over time.

This experiment wants to identify what are the maximum and minimum dilution volume values in the cellstat that allow maintaining it. No other selective pressure is still introduced in the system.

At equal introduction of host cells, the only variable parameter in the volume of cellstat is then the in/out flow, i.e. Dc. Samples of infected or non-infected culture and free pahegemid, at known instant times, have been collected from the cellstat output, to assess the concentration of free phagemid *p* [CFU/mL] against Dc. The experiment has been conducted using a basic prototype: chemostat and cellstat.

Below the values and results obtained.

- *Host cells concentration trend*

Time [h]	**Chemostat** OD_{600nm}		**Cellstat** OD_{600nm}		
0	1.350 ±0.025		---	$\mathbf{D_{Cells}}=0.0h^{-1}$	Bacterial culture growth in chemostat for 18 hours to reach the steady state and reduce its oscillation.
0.5	1.123		---		
1	0.808		---		
3.5	0.560		---		
16	0.535		---		
17	0.520		---		
Experiment starts, Cellstat of $\mathbf{V_c=1.5ml}$ is now connected					
18 ÷ 20	0.548		0.512	$\mathbf{D_{Cells}}=13h^{-1}$	
20.5 ÷ 22.5	0.522		0.610	$\mathbf{D_{Cells}}=6.5h^{-1}$	
23 ÷ 42	0.570		0.621	$\mathbf{D_{Cells}}=3.5h^{-1}$	Contamination starting from 37th hour.

Table 3: the bacterial culture had grown in batch culture overnight, then refreshed and after 5 hours a sample of 54 ml was inoculated into the Chemostat vessel. Here, the culture remained overnight for other 18 hours under equilibrated growth condition, so that, the morning after it was at its concentration steady state. Then, the chemostat has been connected to the cellstat for conducting the experiment.

- *Dependence of free phagemid p (CFU/ml) on dilution D_{Cells} at costant total hosts cells (CFU/ml) $0.548 \leq OD_{600nm} \leq 0.621$*

At t_0 of the experiment, 4µl of original M13 pahgemid supernatant has been inoculated into the cellstat volume of 1.5 ml. In order to facilitate the infection, as suggested by our collaborators, Dr. M. Isalam, Imperial College, the cellstat has been mantained at a temperature of 30°C.

The phagemid carries the gene for the ampicillin resistant, so that, it is possible evaluate the concentration of free phagemid into the cellstat for each dilution rate sampling periodically the output. The mixtures have been then spinned and filtered for extracting the supernatant. This has been finally mixed with uninfected culture from the chemostat and plated on LB+KAN+AMP plates in several dilutions as protocol suggested.

The host cells are instead KAN resistant thanks to the HP plasmid (Tab. 4).

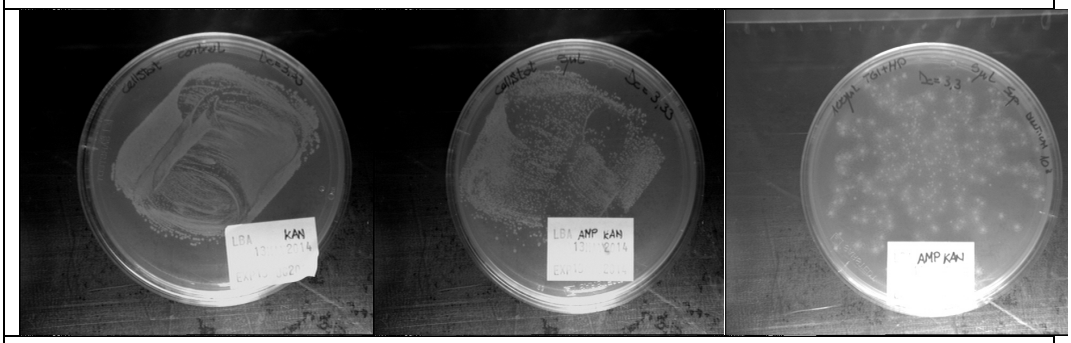
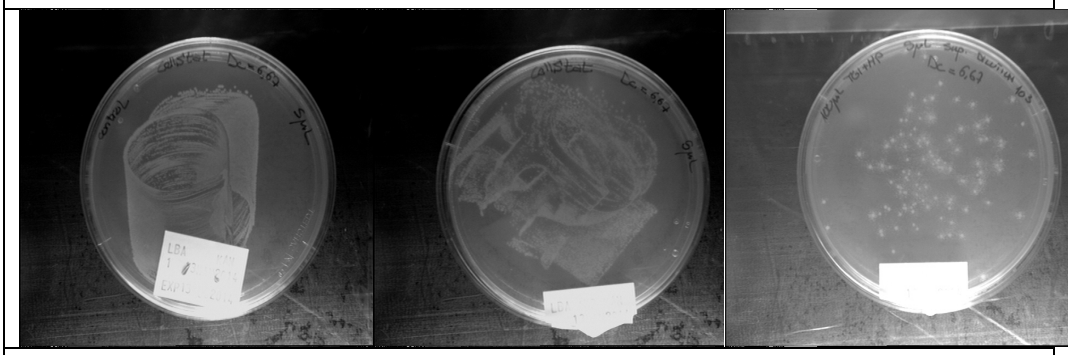
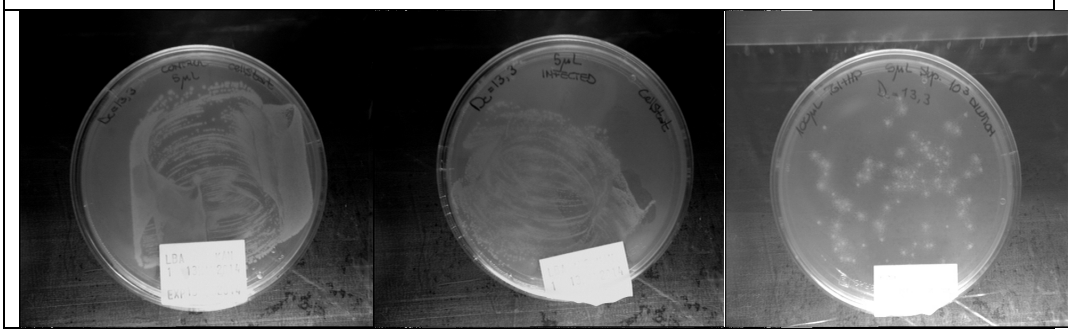
Total cells h_0 on LB+KAN plate	**Infected cells h** on LB+KAN+AMP plate	**Free phagemid p from supernatant** mixed with uninfected chemostat cells and plated on LB+KAN+AMP
$D_{CELLS} = 3.5h^{-1}$		
		
$D_{CELLS} = 6.5h^{-1}$		
		
$D_{CELLS} = 13h^{-1}$		
		

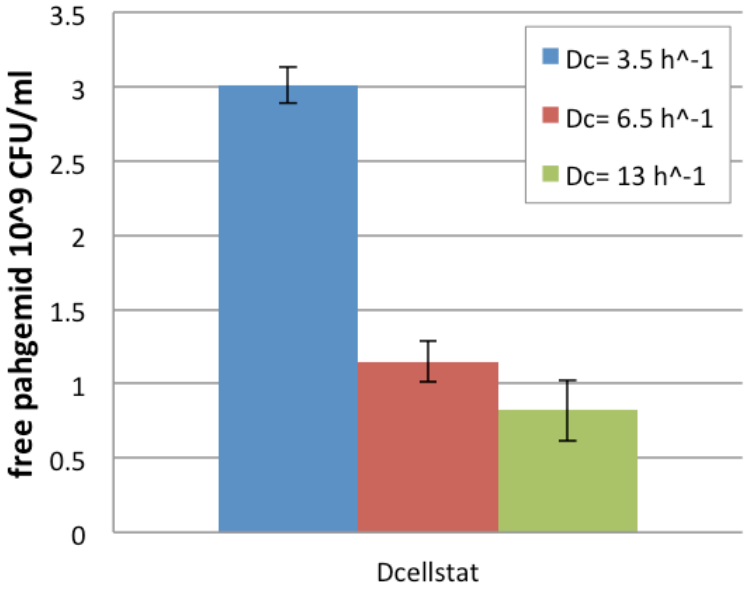
Table 4: pictures of the plates related to total, infected cells and free phagemid concentration p from supernatant for each D_{Cells}. For a preliminary comparison between the amount of total and infected cells, 100µl of mixture has been plated on LB+KAN and LB+AMP respectively. For counting the colonies related to p, 5µl of supernatant has been plated on 100µl of uninfected chemostat culture in several

dilution. Not all the dilution plated are shown.
Controls such as not diluted M13 phagemid original supernatant ($1.5x10^{10}$ CFU/ml) and pure LB (0.0 CFU/ml) on several culture dilutions are here not shown.

Here in table 5, the values of density and colonies from the previous table 3 and plates (Tab. 4). Each $D_{Cellstat}$ rate has been kept fixed for at least 2 hours. During this time every sample, measurement and control has been carried out. The graph 3 shows the trend of free phagemid against $D_{Cellstat}$.

$D_{CELLSTAT}$ [h^{-1}]	OD_{600nm} [±0.025]	Tot Cells **[CFU/mL]** $OD \cdot 8 \cdot 10^8$	Colonies Dilution 10^{-3}			Free Phagemid **[PFU/mL]** Col•Dil•(1ml/0.005ml)
3.5	0.621	$49.6\ 10^7$	323	317	341	**$3.01\ 10^9$**
6.5	0.610	$48.8\ 10^7$	115	121	97	**$1.15\ 10^9$**
13	0.548	$43.8\ 10^7$	96	68	87	**$0.82\ 10^9$**

Table 5: resuming of the results obtained by colonies counting.



Graph 3: plotting of the the trend of free M13 phagemid in suspension related to each dilution rate $D_{Cellstat}$.

4.5.1 Considerations about M13 phagemid experiment

Despite the high starting dilution rate, equal to $13h^{-1}$, no extinction happened during the first two hour of experiment. This could be due to the high density of phagemid in the original stock comparable to $1.5x10^{10}$ CFU/ml. Also, the small amount of free phagemid in suspension seems to suggest that all the

phagemid available into the cellstat is suddenly adsorbed by the uninfected host cells.

This wide range of dilution rate is a significant data regarding the increasing of efficiency of assisted evolution experiment, such PACE, using phagemid for increasing the speed.

However, due to a contamination of the media growth used, here, we cannot confirm that even the smallest dilution equal to $3.5h^{-1}$ could be reasonable for conducting this experiment in long run. This is currently investigated by another parallel project for better understanding the minimum time and starting concentration of phage requested for obtaining a result comparable to the Liu's paper (2014): *A system for the continuous directed evolution of biomolecules.*

- *Biofilm formation*

  Because M13 phage is a filamentous type, in this experiment E. coli F^+ have been used. Already, after 42 hours, biofilm was detectable on the inner surface of the chemostat (Fig. 21).

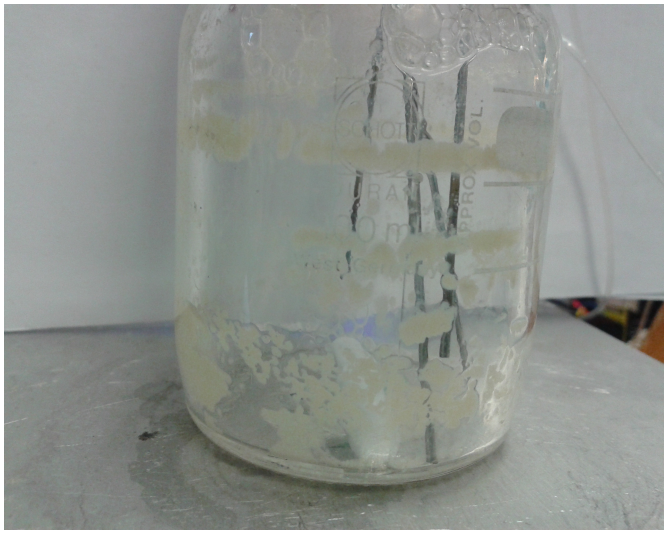


Figure 21: the picture shows the biofilm formation on the inner surface of the chemostat vessel already after 42h. This phenomena on long run experiment affects the fittings condition, causing their clogging and the model parameter, varying the coefficient values.

- *External contamination*

Since when the culture was inoculated into the chemostat for the equilibrated growth, the presence of contamination appeared on the 37th hour LB+KAN+AMP plate control (Tab. 6). Following studies, not showed in this thesis, has confirmed that the origin of the contamination came from the vessel of medium growth, maybe due to an inappropriate sterilization.

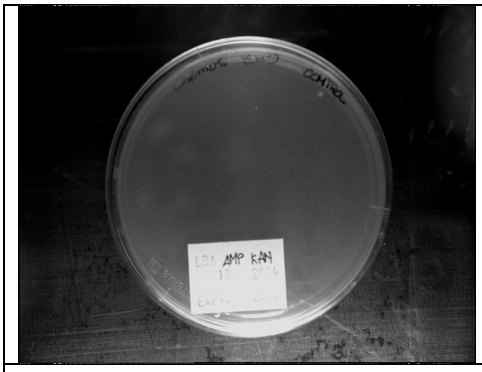
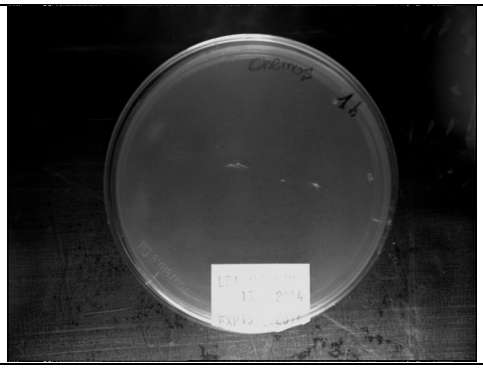
	
t=0	t=1hr
	
t=3.5hr	t=37 hr **contamination**

Table 6: pictures of the plates related to the contamination control. The contamination, AMP+KAN resistant appears starting from the 37th hour. Positive and negative control are not showed.

4.6 Relation between free T7 lytic bacteriophage phage end dilution rate

As investigated in the previous case, also for the bacteriophage T7, the aim of the experiment is to assess which are those values of dilution rate that allow to maintain the viral population without extinction or domination of phage on bacteria. Here indeed, it has considered the phase of the burst due to the lytic

cycle. Moreover, in this study, each dilution has been conducted with independent experiments runs.

So, maintaining fixed the concentration of host cells and the dilution Dc, we have collected samples outgoing from cellstat for three different values of $D_{Cells.}$ To prevent further viral infection of the cells after the sampling, the sample was suddenly filtered obtaining thus, supernatant from free phage in suspension.

The experiment has been conducted using a basic prototype. Chemostat and cellstat are both at a temperature of 37°C. The bacterial culture density has been kept fixed to 0.600±0.025 with a volume of cellstat of 2ml.

For each experiment run, the culture has been inoculated after refresh and maintained overnight under the conditions of continuous culture in chemostat. Just, at t_0 of the experiment, the cellstat was connected to the first stage of the bioreactor and 4µl of bacteriophage from original stock ($3x10^{10}$ PFU/ml) has been added. This means that, at the t_0 of the experiment the ratio between infectious virions to viable cells into the volume, thus the multiplicity of infection[10], was equal to: MOI = 0.125.

Below the values and results obtained estimate the unknown concentration of free phage, comparing the trend of the cellular OD with the values of known concentration from different D_{Cells}, thanks to a 96-well plate reader (Graph 4). The protocol adopted is a modified procedure for the Kinetic Lysis Assays described by Qimron and colleagues in the paper: *Escherichia coli genes affecting growth of T7 bacteriophage* (2006).

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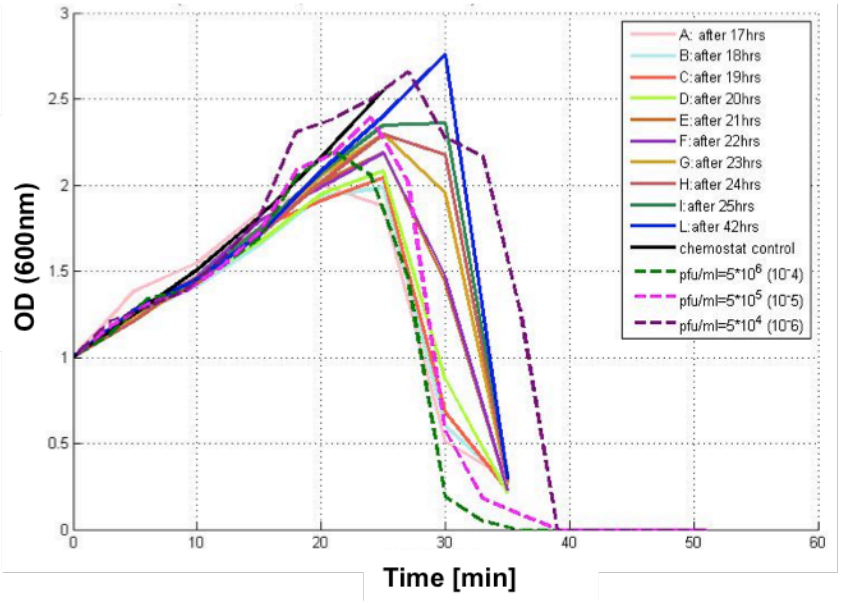
[10] The multiplicity of infection, or simply MOI, is an important parameter to take into consideration in v irology since it was introduced 70 years ago (Ellis et al., 1939). It is the ratio of infectious virions to viable cells in a sample. In other words, the MOI is the average number of bacteriophages per bacterium:

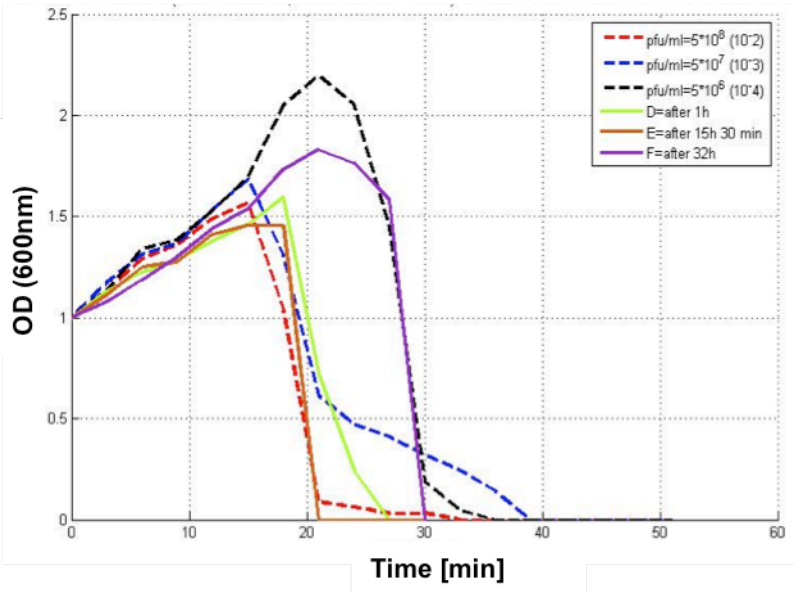
$$MOI = \frac{\left(\frac{PFU}{ml}\right) * ml\ of\ phges}{\left(\frac{CFU}{ml}\right) * ml\ of\ cells}$$

However a MOI interpretation is: The actual number of viruses or bacteria that will enter any given cell is a statistical process: some cells may absorb more than one infectious agent while others may not absorb any. The probability that a cell will absorb *n* virus particles or bacteria when inoculated with an MOI of *m* can be calculated for a given population using a Poisson distribution.

a $D_{Cells} = 3.5\ h^{-1}$ free phage concentration within $10^4 \div 10^6$ PFU/ml



b $D_{Cells} = 2h^{-1}$ free phage concentration within $10^6 \div 10^8$ PFU/ml

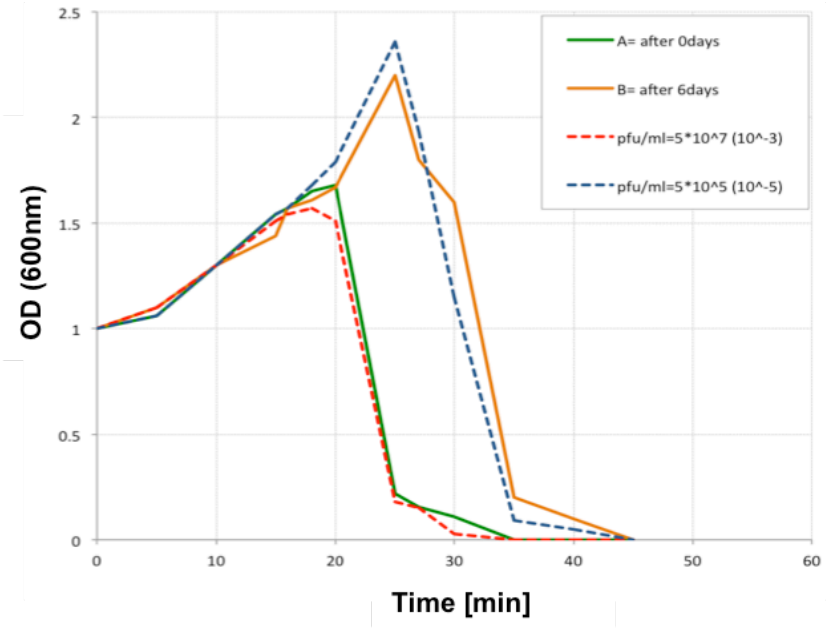


Graph 4: al, **b**l plotting of the cellular OD trends against time, infected with known (dashed line, from stock) and unknown concentration of phage in suspension from samples collected at D_{Cells} 3.5 and $2h^{-1}$. A 96-well plate was used fro the measurements. Each well has been filled with: 0.2ml of LB medium + 0.02ml of the respective phages dilution sample ($10^0 \div 10^{-10}$) + 0.18ml of fresh F^- E. coli culture. Controls are not shown.

4.6.1 First example of long run experiment and free phage trend

Regarding the dilution of D_{Cells} =3h^{-1}, since no contamination appeared, we decided to extend the duration of the experiment over the 42h, up to 6 days. In this case, thanks to the samples collected, we have a graph performance showing which could be the dilution or oscillation of the phage population on periods of time equivalent or close to that corresponding to a PACE experiement.

$D_{Cells} = 3h^{-1}$ free phage concentration within $10^5 \div 10^7$ PFU/ml



Graph 5: plotting of the E. coli OD growth against time, infected with known (dashed line, from stock) and unknown concentration of phage in suspension from samples collected at day 0 and day 6 at D_{Cells}= 3h^{-1}. Controls and intermediate samples values are not shown.

4.6.2 Considerations about T7 phage experiments

Even repeating the experiment three times, the protocols followed and the technical solutions adopted, have demonstrated exceptional strength and reliability producing high quality data (Tab. 6). Indeed the hypothesis of remaining within a range of dilutions, related to the temporal effects of the lytic cycle on a cellular culture in static conditions in order to avoid phage extinction or domination, allowed to successfully support the population.

D_{CELLST} [h^{-1}]		T7 Free Phage [PFU/mL]
3.5	(17')	$\mathbf{10^6 \div 10^8}$
3	(20')	$\mathbf{10^5 \div 10^7}$
2	(30')	$\mathbf{10^4 \div 10^6}$

Table 6: resume of the result obtained by plaque assays counting for each dilution tested

The previous table shows which are the orders of magnitude of free phage [PFU/ml] for each D_{Cells} tested, confirming that:

- Different flux have an identifiable effect on the concentration in suspension;
- At steady state, the phage population reduces its oscillations in terms of concentration, within distinct density values;
- It is possible to maintain a phage population, T7 type for long run experiment such as PACE technique requires.

- *Biofilm formation*

Because T7 phage is a virulent type, in this experiment E. coli F^- have been used. Despite a run of 6 days, no significant biofilm formation was detectable on the inner surface of the chemostat (Fig. 22).

Figure 22: picture of the vessel used as chemostat after 6 days of run. No detectable biofilm formation appears. Anyway, it is visible the inevitable edge

formed by cell culture.

- *External contamination*: negative to all the controls.

4.7 A preliminary experiment to demonstrate homologous recombination of T7 phage in continuous culture.

Homologous recombination in continuous culture demonstrates several qualities of the second bioreactor prototype. This setup allowed, indeed, to switch cultures during a run, enabling a commutation from an homologous recombination strain to a selection one after 60 min (Fig. 23).

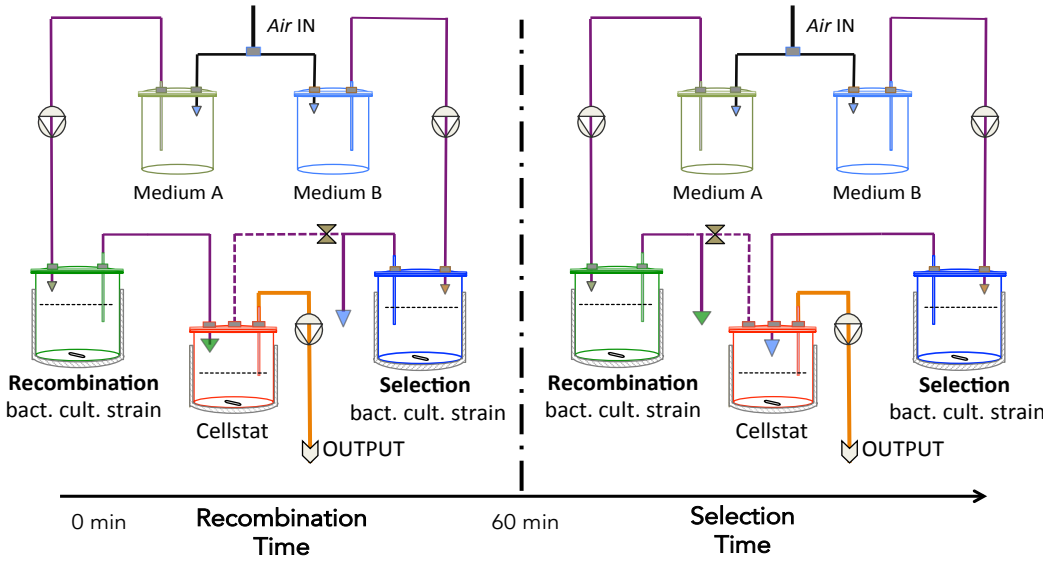


Figure 23: logic scheme of the bioreactor configuration used for feed periodically switched into common cellstat.

As stated by the previous chapter 3.4.2, T7 phage is thus solely able to reproduce if it gains *cmk* and *trxA* genes through recombination. The selection process itself illustrates the amplification of extremely small populations of the desired recombined phage after just 153 min, from undetectable levels (Table 7).

Phage Sample Time (Min)	PFU/ml	
	Recombination Strain	**Selection Strain**
57	5.5×10^6 ($\pm 1.16 \times 10^6$)	$<10^1$ (NA)
73	1.16×10^6 ($\pm 0.66 \times 10^6$)	$<10^1$ (NA)
153	3.1×10^4 ($\pm 0.7 \times 10^4$)	1.9×10^4 ($\pm 0.44 \times 10^4$)

Table 7: plaque assay PFU (plaque forming units) results of phage sample taken from the bioreactor at three given times. The phage samples were plated on both the recombination strain and the selection. Standard deviation shown in parenthesis. *Courtesy of J. Hassal, University of Warwick.*

Chapter 5

5 Conclusion and future directions

The prototypes here presented confirm the possibility to extend the study, under different aspects, of new biological concepts limited before by the need to use complex and expensive apparates. Also, thanks to its design and the use of solutions commonly found in a laboratory, this original device facilitates the approach to the exploitation of complex techniques such as PACE. The data and values obtained, indeed, prove that a certain flexibility in the choice of parameters, which contribute to increasing the efficiency of the evolutionary process, ensures a wide application for different experiments that need bacterial cultures properly engineered and maintained separately in continues culture to sustain a viral population, such as bacteriophages. Thus, as stated, simply by adjusting the dilution and setting the termperature, it is possible to get an acceleration of evolution up to 140 rounds per day maintaining under steady optimal biological conditions.
The developed system also proves to be a robust and simple instrument that can be used by researchers not directly involved in its development, after a simple and short training.

If considered as a module, the bioreactor made by chemostat and cellstat, or phage reactor, can be duplicated to obtain arrays. This aspect makes is the foundation for all future developments concerning the project Evoprog whose main purpose is to construct a machine that using existing phage technology and synthetic regulation is able to produce biomolecules or biocomputational functionality two orders of magnitude faster than conventional techniques, while consuming fewer consumables. “In its core, living matter will be subject to combinatorial search algorithms that will exploit large numbers of small, separate, bacterial populations. Each one will contain phage that evolves under different custom fitness selections. The different phage will then be recombined according to combinatorial optimization strategies.” (Dr. A. Jaramillo)

To date the system, based on the concept of continuous culture, is subject to restrictions introduced by the process itself to this scale. The continuous culture of cells suffers indeed by problems related to the biofilm and spontaneous evolution of bacteria.
The development of biofilm into tubings or volume surfaces which are in direct contact with the culture produces an undesired effect in the system introducing two types of problems:

- Tubing and needles clogging
- Just for temperate phage its presence affects the condition of the evolutionary model because as if it appears in cellstat, or downstream, the number of phage is suffering from those produced by the biofilm.

This problem does not appear instead for the case of lytic phage, as they would remove it, each time that an infected cell remains inside the vessels for more than the time of the burst. After that, as consequence, it would lyse creating a temporary excess of phage.
To overcome the effect of biofilm, it is possible to adopt different strategies like the use of non-toxic cleaners like Polysorbate 20, or using technical solutions that provide the switch of the culture in other containers to allow the rinsing [40], or even replacing periodically the same culture but with different antibiotic resistance, which flows into the system, without effecting the viral population.
Concerning the spontaneous evolution, it is a predictable effect but still under study, for example in the laboratory of R. Lensky and the *E. coli* long-term evolution experiment Project, as the bacteria seek to increase fitness under the conditions of the system in which they grow.

The prototype object of this thesis, as well as participating in the development of the project Evoprog, can be used, thanks to its modular and easy construction, for projects which ultimate goal is increasing the specificity of the molecules.

For example the development of procedures in order to produce engineering synthetic bacteriophage cocktails against specific bacteria (Fig. 24) or a

system that can create different ecosystems in co-culture for the continuous screening of the viral population and its characteristics.

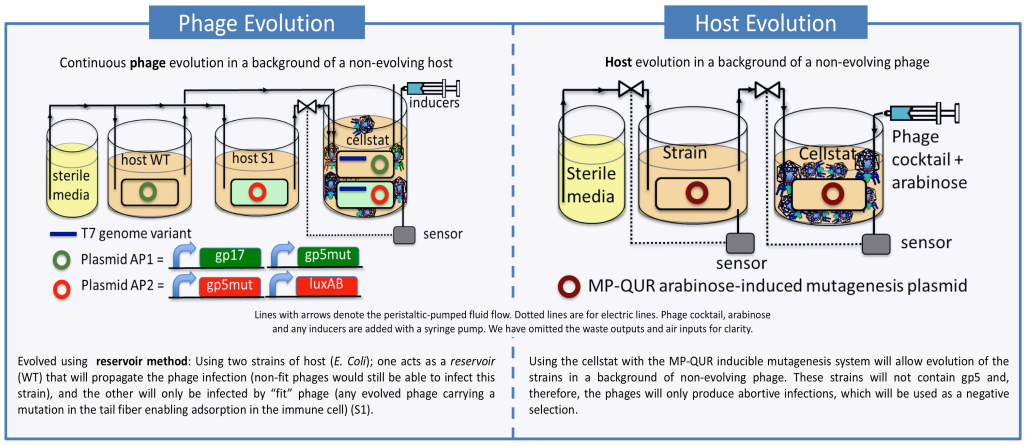


Figure 24: Engineering synthetic bacteriophage cocktails. *J. Hassal, F. Polesel, A. Jaramillo; CDT Poster, University of Warwick 2014*

One of the next steps would be increase of the level of automation of the system by introducing additional valves, controls and sensors. For example, a sensor that is able to determine the rapport between host cells and phage in real time, and then actively vary the ecosystem.

Another aspect we would like to develop is the scalability and miniaturization system. Regarding to this two applications for patents have been filed.

Eventually, a commercial solution is now being studying by the Warwick University and Jaramillo's Lab.

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Appendix A: equipment list

Fluidics:

- Bioprobe flask, 250 mL – Bellco glass – turbidostat/chemostat
- 2 x 25 mL Bioprobe flask – Bellco glass – cellstat(s)
- 3 x Corning scholar 171 magnetic stirrer – Thermo Fisher Scientific – stirs bioreactors
- Watson Marlow multichannel programmable pump
- Masterflex L/S economy variable drive – Cole Parmer, Inc – programmable pump
- 3 x large flasks – any manufacturer – LB source, cellstat/chemostat/turbidostat waste
- Laboratory film (parafilm) - covering open vessels, e.g. LB source, waste vessels, etc
- FEP, TYGON and SILICON tubing, various diameters – Dolomite centre, microfluidic solutions – fluid transfer
- Non-sterile needles, blunt end – cadence science – fluid withdrawal
- Stainless steel needles and luers fittings various gage and length - SigmaAldrich
- Masterflex plot silicone tubing, 1.42 mm ID – Cole Parmer – tubing to operate with the peristaltic pumps
- GL45, GL32, GL25 open caps – Bellco glass – lids for 1l, 50 ml, 25 ml vessels, respectively
- GL45, GL32, GL25 septa – Bellco glass
- 0.2μm single use filter unit – Sartorius stedim

Electronics:

- Arduino Uno version 3 microcontroller – Arduino
- Air pump AP 200 - Wave point air pump
- Acquarium heater Easyheater 25- Aquael
- EC4010M12C Pc fans + neodymium magnets ø 6mm
- Spare parts and components

- Acrylic box
- 3D printed holders and lids