Universitat Autònoma de Barcelona

ANALYSIS OF THE BACTERIOPHAGE P22 VIRAL SPREAD WITHIN BACTERIAL POPULATIONS AND ITS CHARACTERIZATION AS IMMUNOBIOSENSOR

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PhD Thesis

submitted by

MARIA ESTER RAMÍREZ VÁZQUEZ

to the Universitat Autònoma de Barcelona (UAB)
in fulfilment of the requirements for the degree of
Doctor of Philosophy
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Approval of the Thesis Director,

Prof. Dr. Antonio P. Villaverde Corrales

Bellaterra, November 2015

Als meus pares,
pilar fonamental de la persona que sóc.
A les meves filles,
motor que em fan créixer dia a dia.
I al meu company de viatge,
amb el que l'aventura sempre és al camí.

CONTENTS

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1 - INTRODUCTION

1.1. BACTERIOPHAGES

Although Ernest Hanbury Hankin already reported in 1896 that something in the waters of rivers in India had unexpected antibacterial properties against cholera (Hankin, 1896), bacteriophages were independently discovered by Frederick Twort (Twort, F.W., 1915) and Felix d'Hérelle (D'Hérelle, 1917; Summers, W.C., 1999).

Bacteriophages, also commonly referred as 'phages', are viruses that infect bacterial cells (Sulakvelidze, A., 2013). The phages as the rest of viruses are simple structures consisting usually of two basic components (Schlesinger, M., 1934):

- **nucleic acid**: double- or single-stranded RNA or DNA, from 3-5 genes in simple phages to 100 genes in complex phages and
- **a protein envelope or capsid** to protect the nucleic acid from nucleases (the simplest phage have many copies of only one or two different proteins while more complex phages may have many different kinds). Some of them they have lipids as components of the envelope or of a particular lipid wall (Ackermann 2003).

The phages don't have many of the enzymes and structures necessary for reproduction, and therefore they cannot reproduce by theirself. So they must infect bacterial cells to use the host's metabolic machinery to synthesize virion components and make new copies of themselves inside bacterial cells (Sulakvelidze, A., 2013; Wang, J.P, et al., 2013). In this way, phages are obligate parasites of bacteria, as the survival of viruses is totally dependent on the continued existence of their host (Orlova, E.V., 2012).

Phages are arguably the oldest (3 billion years old, by some estimates) and most ubiquitous (10^{30}-10^{32}) known organisms on Earth (Whitman, W.B. et al., 1998; Sulakvelidze, A., 2013; Mc Grath S. and van Sinderen D., 2007), ten times more numerous than bacteria (Hendrix, R.W., 2002; Hanlon, G.W., 2007). The ability of phages to survive under unfavorable conditions, such as temperature, acidity and salinity, is highly diversified (Jonczyk, E. et al., 2011). Phages can be found in all environments where bacteria grow, even extreme: in the Sahara, hot springs, polar inland waters (Breitbart, M. et al., 2004, Säwström, CH. et al., 2008), in ground and surface water, soil, food, sewage, and sludge (Lucena, F. et al., 2006; Yoon, SS. et al., 2002). They have also been isolated from human and animals, from feces, urine, saliva, spit, rumen, serum, etc (Gantzer, Ch. et al., 2002; Bachrach, G. et al., 2003). Phages are able to penetrate different organs and tissues, including the central nervous system, and are a part of intestinal flora together with their bacterial hosts (Frenkel, D. and Solomon, B., 2002; Kameyama, L. et al., 2001).

Phages are classified by the International Committee on Taxonomy of Viruses (ICTV) (Mc Grath S et al., 2007), according to phage morphology and the nature of the nucleic acid (Van Regenmortel, M.H.V. et al., 2000; Ackermann, H.W., 2003; Ackermann, H.W., 2006; Ackerman, H.W., 2009), in 14 officially accepted families and at least five other potential families awaiting classification. More than 5500 phages have been examined in the electron microscope (Ackermann H.W., 2007).

Most phages range in size from 24-200 nm in length. Although viruses are extremely diverse in their life cycles and infectious mechanisms, the vast majority of phages share a common structure, consisting of heads and tails (96%) and contain linear double strand DNA (dsDNA):

- The head or capsid is a spherical protein capsid composed of many copies of one or more different proteins and that encloses condensed nucleic acid (Herriot, R.M., 1951), acting as the protective covering.
- The tail is a hollow tube, which helps the phage attach to its host and through the nucleic acid passes during infection. The size of the tail can vary.

Tailed phages (which are non-enveloped and contain linear dsDNA) fall into three families, which constitute the order Caudovirales (Ackermann, H.W., 2009):

- *Myoviridae* (25%) (with contractile tails, where the tail is surrounded by a contractile sheath, which contracts during infection of the bacterium. At the end of the tail, there is a base plate and one or more tail fibers attached to it, involved in the binding of the phage to the bacterial cell) (T4, T2, Φ gspC, CP-51)
- *Siphoviridae* (61%) (with long, but simple and non-contractile tails) (λ, SPP1, T5, HK97)
- *Podoviridae* (14%) (with short and non-contractile tails) (P22, T3, T7, ϕ29).

Only 190 phages (3.6 %) are filamentous or pleomorphic (Ackermann, H.W., 2007; Hendrix, R.W., 2002).

Also phages with single-stranded DNA (ssDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) are a minority (Ackermann, H.W., 2009).

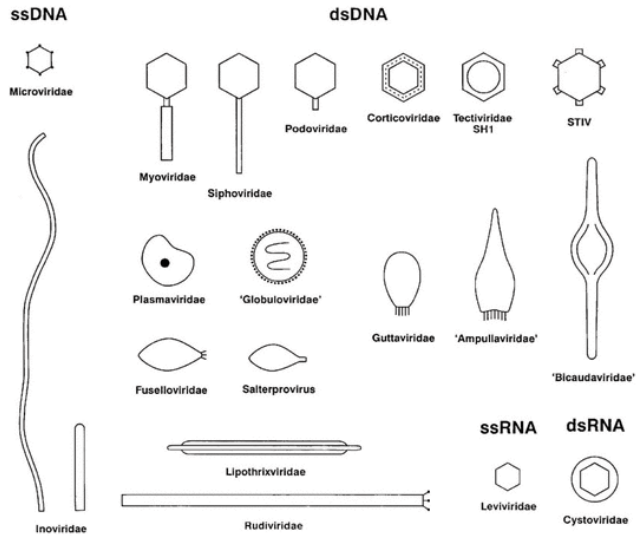


Figure 1 - Schematic representation of bacteriophage families (Ackermann, H.W., 2009).

1.2. PHAGE SPREAD: LYSIS AND LYSOGENY

1.2.1. Phases of Phages' Replication

Ellis and Delbrück performed in 1939 a very simple experiment called one-step-growth-experiment (Ellis, E.L. and Delbrück, M., 1939; Cann, A.J., 2015). This was the first experiment to demonstrate the three essential phases of virus replication:

1. Adsorption (attachment) of the phage on the bacterial cell (Murray, A.G. and Jackson, G.A., 1992; Abedon, S.T. et al., 2001). It may be further divided into:

1.1. Diffusion-mediated extracellular search.
1.2. Collision between phage and bacterium.
1.3. Attachment between phage and susceptible bacteria.
    1.3.1. Reversible attachment: the first step is the interaction between the Long Tail Fibers (LTF) of the phage and specific receptors of the bacterial cell surface (such as proteins, lipopolysaccharide (LPS), pili and lipoproteins (Rakhuba, D.V. et al., 2010; Abedon, S.T., 2006; Braun, V. and Hantke, K., 1997).
    1.3.2. Irreversible attachment: The recognition signal sent through the LTFs to the baseplate unravels the short tail fibers (STF) that bind irreversibly to the cell surface. The baseplate changes conformation and this results in the contraction of the sheath and the hollow tail fiber is pushed through the bacterial envelope.
1.4. Nucleic acid uptake into the bacterial cytoplasm. When the phage has gotten through the bacterial envelope, the nucleic acid from the head penetrates through the hollow tail and is injected into the bacterial cell. The remainder of the phage, the capsid, remains on the outside of the host cell as a "ghost".

Figure 2 - Adsorption of the phage on the bacterium.

2. Latent Period (time around 20-25 min between adsorption and the lysis). Replication of the virus genome occurs within the bacterial cell, without increase in extracellular phage (Doermann, A.H., 1952).

2.1. Eclipse period: It begins after the nucleic acid is injected and finishes when the first phage is completed inside the bacterium. The eclipse period can be:

2.1.1. prevegetative in the sense of immediately proceeding phage-progeny maturation: LYTIC Cycle

After phage genome entry into the cell, as bacteria have specific mechanisms to protect themselves against phage's infection ("restriction/modification" systems which depend on the recognition and destruction of foreign DNA), many phage genomes are degraded and destroyed. Surviving phage genomes take over the host biosynthetic machinery, induce switching of the protein machinery of the host bacterium to achieve the intracellular synthesis of virus components. Structural proteins (head, tail) that comprise the phage as well as the proteins needed for lysis of the bacterial cell are separately synthesized. No infectious phage particles can be found either inside or outside the bacterial cell. This cycle, where phages immediately proceeding phage-progeny maturation and death of the host cells, is called lytic cycle. The phages which are only following the lytic cycle are called Virulent phages (e.g. T-phages of *Escherichia coli (E. coli)*, such as phage T4). Lysis involves a tradeoff between maximizing per-infection phage productivity and minimizing the phage generation time (Abedon, S.T. et al., 2001). So long as virus particle remains inside an infected bacterium then it is not free to acquire a new host.

2.1.2. temporarily or greatly extended, as observed, respectively, with pseudolysogeny and lysogeny: LYSOGENIC Cycle

A lysogenic infection occurs when the viral genome becomes integrated into one of the host cellular replicons (chromosome, plasmid, etc.) (Williamson, S.J. et al., 2001). In this quiescent or dormant state most of the phage genes are not transcribed. The phage genome exists in a repressed state within bacterial cells, so called prophage because it is not a phage but, although there isn't a productive infection, it has the potential to produce progeny phage under certain circumstances. The prophages replicate along with the host cell and are passed onto daughter cells. The cell harboring a prophage (termed a lysogen or lysogenic bacteria) is not adversely affected by the presence of the prophage and lysogenic state may persist indefinitely with minimal viral gene expression. This cycle is called lysogenic cycle (Lwoff, A., 1953). Prophages remain dormant, following the lysogenic cycle until the lytic cycle is activated. Phages which can either multiply via the lytic cycle or via the lysogenic cycle are called Lysogenic or Temperate phages. (Guttman, T. et al., 2005). Then temperate phages establish a persistent infection of the cell without killing it (lysogenic cycle) until the lytic cycle is activated. A very well-studied example of a temperate phage is phage λ of *E. coli* (Kourilsky, P., 1973; Van Regenmortel, M.H.V, 1990; Kenneth, M.A. et al., 2011).

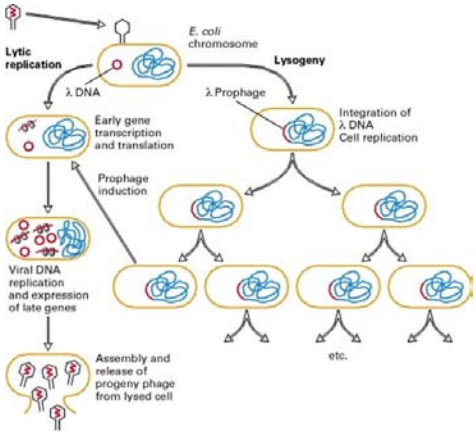


Figure 3 - Two cycles of bacteriophage replication: Lysis vs Lysogeny

2.2. a period of phage-progeny maturation (assembly). The assembly of phage components into mature infective phage particle is known as maturation. The nucleic acid is packaged inside the head and then tail is added to the tail. Then mature infectious phage particles accumulate within the cell.

3. Phage Progeny Release. To make so many new phages, it is required nearly all the resources of the cell, so the bacteria becomes very weak. On the other hand, in the most cases due to the accumulation of the phage lysis proteins (Wang, I.N. et al., 2000), the bacteria begin to lyse and dies. Then, the new synthesized and mature virions are released into the extracellular space (Doermann, A.H., 1952; Orlova, E.V., 2012), ready to find new hosts to infect. The progeny release could occur by other mechanisms different to the lysis and host cell death, such as in the case of filamentous phages, which extrude their phage progeny across the host cell envelope.
The number of phage particles released per infected cell, called the **burst** size, varies with the particular phage and the particular host cell, may be as high as 1000 (Moat, A.G. et al., 2002). Lytic phages are enumerated by a plaque assay. A plaque is a clear area, which results from the lysis of bacteria. Each plaque arises from a single infectious phage. The infectious particle that gives rise to a plaque is called a pfu (plaque forming unit).

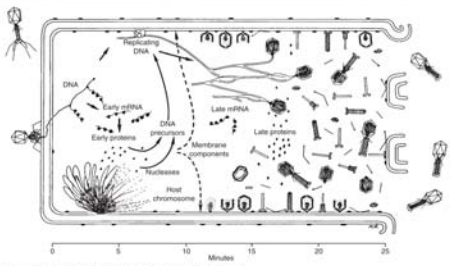


Figure 4 - Overview of the phage lytic cycle (T4 life cycle)
(Mosig, G. and Eiserling, F., 2006; Mathews, C.K. et al., 1983).

On the other hand, phage decay, that would be equivalent to virion death, or inactivation or loss of titer (Douglas, J., 1975), implies that virion populations cannot survive indefinitely in the absence of sufficient densities of susceptible bacteria (Williams, S.T. et al., 1987; Suttle, C.A., 2000; Suttle, C.A. and Chen, F., 1992; Stewart, F.M. and Levin, B.R., 1984).

1.2.2. Decision between Lysogenic and Lytic Growth

The lytic cycle can be activated depending on the environmental conditions (Echols, H., 1972; Herskowitz, L. and Hagen, D., 1980; Ptashne, M., 1992):

- in a spontaneously way by CI repressor / Cro protein balance:
    - if environmental conditions favour the production of a phage-encoded repressor (the product of the *cI* gene of the prophage (Jacob, F. and Monod, J., 1961), the lytic genes are repressed by the CI repressor and established the lysogeny.
    - if environmental conditions favour the production of Cro protein, which binds to the same site as CI repressor binds, the phage will activate the lytic cycle (Svenningsen, S.L. et al., 2005; Ptashne, M., 1987).
- in an induced way, by SOS system activation through RecA protein activation.

It has been shown that when DNA is damaged by UV irradiation, mitomycin C, etc or the DNA replication is inhibited (Walker, G.C., 1985; Reddy, M and Gowrishankar, J., 1997), the bacterial SOS system is activated with the consequent generation of mutations (Walker, G.C., 1985; Weinbauer, M.G. and Suttle, C.A., 1999; Taddei, F. et al., 1995; Craig, N.L. and Roberts, J.W., 1980; Roberts, J. and Devoret, R., 1983; Walker, G.C., 1987; Defais, M. et al., 1971).

The SOS system involves the coordinated activity of more than 20 gene products, which promotes DNA repair and prevent cell division until replication of the cell crhomosome is restored (Litte, J.W. and Mount, D.W., 1982; Kenyon, C. and Walker, G., 1980).

When DNA is damaged by UV irradiation, mitomycin C, etc. or the DNA replication is inhibited, single-strand oligonucleotides are formed as breakdown products (Walker, G.C., 1987). The binding of RecA protein to single-stranded DNA reversibly activates a co-protease activity of the RecA protein (Ackermann, H.W. and Dubow, M.S., 1987; Duwat, P., et al., 1995).

RecA stimulates the self-cleavage of the LexA repressor of numerous SOS genes (Kim, B. and Little, J.W. (1993), and of the CI repressor of λ and P22 (Phizicky, E.M. and Roberts, J.M. 1980; Kim, B. and Little, J.W., 1993).

When LexA is cleaved in response to DNA damage, the proteins in charge to repare DNA are synthesized. In λ or P22 lysogens, activated RecA is also capable of mediating the cleavage of λ CI repressor, resulting in prophage induction (Gottesman, M. and Oppenheim, A., 1994).

1.2.3. Phases of the Bacterial Life Cycle

The typical bacterial growth curve (Hall, B.G. et al., 2014) has the following phases:

1. Lag Phase

Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity. It includes the time during which growth accelerates.

2. Exponential Phase

During normal exponential growth, *E. coli* cells undergo cycles of cell growth and division in which daughter cells are virtually identical to the mother cell.

Under exponential growth conditions, a major cellular response to perturbation of DNA metabolism is the induction of the SOS system. Besides DNA repair, recombination and the fidelity of replication, the *E. coli* SOS response affects cell division, transposon mobility, and horizontal gene transfer (Taddei, F. et al., 1995).

3. Stationary Phase

The growth of bacteria in the natural environment is very often limited because nutrients are quickly exhausted, leading to starving conditions and, subsequently, they reach a point when the growth rate decreases, indicating the onset of stationary phase.

In order to insure their survival, bacteria should be able to make an orderly transition into stationary phase such that the cell cycle is not arrested randomly. In addition, bacteria must also be able to remain viable during prolonged periods of starvation and to exit stationary phase and return to the exponential cell cycle when starvation is relieved.

3.1. Entry into Stationary Phase

The entry into stationary phase is a transition period beginning at the point in the exponential phase when all cellular parameters cease increasing at equal rates, i.e. DNA, protein, and total cell mass no longer increase together, and continuing until the time when no further increase in cell number is detected (Kolter, R., 1993).

In cultures of Gram-negative bacteria, the transition from the exponential growth phase to the stationary phase is accompanied by dramatic changes in the cell metabolism that allow maintenance of cell viability under nutrient starvation (Ishihama, A., 1997; Kolter, R., 1993).

- cells becoming smaller and more spherical as a result of induction of the *bolA* gene Aldea, M. et al., 1989).
- the cytoplasm becomes condensed while the volume of the periplasm increases,
- the composition of the cell membrane is altered to produce a less fluid membrane, reflecting the need for protection from stressful environments (Alexander, D.M. and St. John, A.C., 1994),
- the nucleoid becomes condensed by replacement of some DNA-binding proteins and
- there are marked changes in the pattern of global gene expression.

  Approximately 1000 genes highly expressed in the exponentially growing *E. coli* cells are mostly turned off or markedly repressed in the stationary phase cells and instead, a set of 50-100 genes that are repressed in the growing cells begins to be expressed upon entry into the stationary phase. In the case of *E. coli*, it has been shown that the transposase of IS*1* is capable of inducing a host SOS response (Lane, D. et al., 1994). Induction of the SOS response during growth in a rich medium was seen only when cells approach stationary phase. This appears to be not so much a feature of entry into the stationary phase itself as an effect of reduced growth rate. So slow or decelerating growth, might promote the induction of SOS signals at several levels (Lane, D. et al., 1994). It has been

shown a cAMP-dependent SOS induction and mutagenesis in resting bacterial populations, in the absence of exogenous sources of DNA lesions (Taddei, F. et al., 1995). It seems that an increased genetic variability of resting bacterial populations can increase their fitness (Sonti, R.V. and Roth, J.R., 1989). It was found that the *recA* gene expression increases between the early exponential growth phase and the following days. The cAMP-dependent SOS induction between the early exponential growth phase and the following days is consistent with the increase in RecA protein levels and suggested that SOS induction occurs after the end of the exponential growth phase when cell enter the stationary phase (Taddei, F. et al., 1995). Additionally it has been shown a relationship between external glucose concentration and cAMP levels inside *E. coli*. Growth in minimal medium with micromolar glucose results in 8- to 10-fold higher intracellular cAMP concentrations than observed during growth with excess glucose (Notley-McRobb, L. et al., 1997). The function of this increased extracellular cAMP could be that cAMP could serve a cell-to-cell signalling function within a bacterial colony. The extracellular cAMP concentration in a bacterial colony would be predicted to reach much higher levels than in liquid culture where cAMP is diluted in a much larger volume. It was shown that the cAMP concentration in the bacterial host regulates the viral decision between lysogeny and lysis (Hong, J. et al., 1971).

3.2. Maintenance of Viability

After several hours in stationary phase, the stationary-phase bacterial cells complete the developmental process that results in the resistances. Afterwards their metabolic activity is greatly reduced. A priori, there is no reason to expect that any metabolic activity remains in these cells in the subsequent days of starvation. However, starved gram-negative cells are not truly dormant - they remain metabolically active even after many weeks of starvation (this sharply contrasts with the dormant state of the spores produced by many gram-positive organisms).

3.3. Exit from Stationary Phase

Starved cells respond rapidly to the addition of fresh nutrients, increasing in size relatively soon after the addition of nutrients, while the initiation of DNA synthesis lags.

4. Decline/Death Phase

If there isn't addition of fresh nutrients, the number of viable (linving) cells will decline as the population dies.

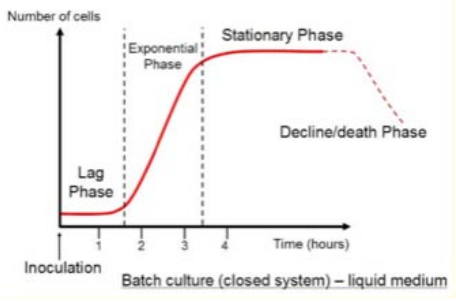


Figure 5 - The typical bacterial growth curve.

1.3. P22 PHAGE

1.3.1. General Background

Phage P22 infects smooth (O-antigen surface polysaccharide carrying) strains of *Salmonella typhimurium (S. typhimurium)* (Levine, M., 1972, Prevelige Jr., P. E., 2006). Phage P22 is a dsDNA temperate phage and is a prototypical representative of the *Podoviridae* family (Orlova, E.V., 2012). Many *Podoviridae*, for example phages T7 and phi29, even though their virion morphologies are similar, have very little if any DNA similarity with P22. Relatives with similar genomic transcription patterns and life cycles include phage λ and all the other lambdoid phages (Casjens, S. 2000; Poteete, A.R., 1988; Susskind, M.M. and Botstein, D., 1978).

Phages from the *Podoviridae* family are characterized by a short base plate or tail structure, incorporated into one of the 5-fold vertex of the icosahedral phage head (Poteete, A.R., 1994). They use their small, tooth-like tail fibers to enzymatically degrade a portion of the cell membrane before inserting their genetic material. On the other hand, phages from the *Podoviridae* family may be extremely resistant to a dry environment (desert sands) and may survive large temperature fluctuations.

The bacteriophage P22 was isolated by Zinder and Lederberg (Zinder N. and Lederberg, J., 1952) a half century ago, and was immediately put to work by *Salmonella* bacterial geneticists because of its unusual DNA packaging properties. It was the first generalized transducing phage to be discovered: a small fraction (2 %) (Ebel-Tsipis, J. et al., 1972) of its virions carry a fragment of the host DNA instead of phage DNA, and this host DNA can be delivered into a host cell (Lobocka, M. and Szybalski, W.T. (eds), 2012). The earliest studies concentrated on regulation of lysogeny. Levine's work on clear-plaques mutants of P22 showed that establishment of lysogeny is regulated by a group of linked genes, only one of which is required for the maintenance of lysogeny (Levine, M., 1957; Levine, M. and Curtiss, R., 1961) and that these genes have a sequential order of action (Levine, M. and Smith, H. O., 1964; Smith, H. O. and Levine, M., 1964). These studies, together with Kaiser's parallel studies of clear mutants of coliphage λ (Kaiser, D., 1957), led to intensive use of P22 and λ in studies of gene regulation at the molecular level.

P22 Structure

The mature P22 virion is an icosahedral protein head (ca. 60 nm in diameter), containing the packaged phage DNA and a short baseplate, sometimes referred to as the tail (ca. 20 nm wide) (Sauer, R.T. et al., 1982; Casjens, S., 1979). The baseplate consists of a central core around which are arranged six spikes (Anderson, T. F., 1960; Botstein, D. et al., 1973; Israel, J. V. et al., 1967). A thin spike or fiber (ca. 20 nm long) emanates from the center of the baseplate. Phage heads lacking baseplates can easily be prepared (Israel, J. V. et al., 1967); these show a small neck and the spike only (Botstein, D. et al., 1973). The P22 virion consists of about equal amounts of DNA and protein.

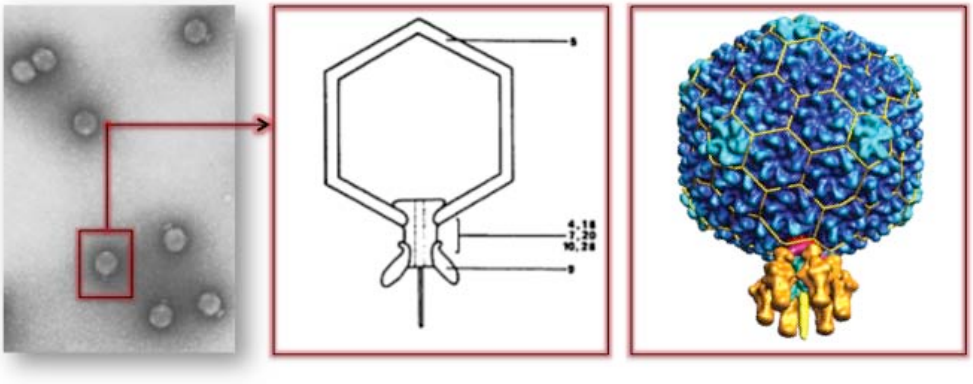


Figure 6 - P22 phage particles
Left: Negatively stained P22 phage particles. (King, J. et al., 1976).
Center: Phage P22 diagram with location of structural proteins (Eiserling, F.A., 1979; Ackermann, H. and Berthiaume, L. (1995)).
Right: Three-dimensional reconstruction of the particles made from cryo-electron micrographs (Thuman-Commike PA et al., 1996; Teschke, C et al., 2003).

P22 DNA

In 1968, Gough and Levine showed that the genetic map of phage P22 is circular (Gough, M. and Levine, M., 1968). However, when the P22 genome is integrated as a stable prophage into the *Salmonella* chromosome, the genes assume a unique linear order (Chan, R. K. and Botstein, D., 1972; Smith, H. O. and Levine, M., 1965). P22 DNA is a single molecule of linear, double-stranded DNA, ca. 28 x 10^6 in molecular weight, which is about 42 kb in length (Casjens, S. and Hayden, M., 1988; Casjens, S. 2000) and has blunt ends. The genome of P22 has been sequenced (Casjens, S. et al., 1989; Eppler, K. et al., 1991; Pedula, M. et al., 2003; Sampson, L. and Casjens, S., 1993) and 65 genes have been anotated (Prevelige Jr., P. E., 2006). The complete genome sequence is available with the accession number AF217253.

The most striking observation about the genetic organization of the phage is that related functions are clustered: DNA replication, lysis and head assembly. The second striking fact about the genetic organization of phage P22 is its similarity to that of coliphage λ.

P22 Proteins

The P22 procapsid consists of:

- 420 copies of the coat protein **gp5** (it's the major component of the head),
- an internal core containing approximately 300 copies of the scaffolding protein **gp8** (Fuller, M and King, J., 1982),
- 10 to 20 copies of each of three pilot proteins: **gp7, gp16 and gp20**. The pilot proteins are believed to reside at one or all of the vertices (Thomas, D. and Prevelige, P., 1991). Both the pilot proteins and the portal complex are required for infectivity but not for procapsid assembly (Botstein, D. et al., 1973).

- and a unique multi-subunit gene 1, **gp1**, portal complex, that is located at one of the vertices of the icosahedral coat protein shell. This is the site DNA entry during maturation and DNA exit during infection (Bazinet, C. and King, J, 1985; Prevelige, P. E., 2006).

The short and non-contractile tail, used by the P22 phage to adsorb to the host cell surface, consists of:

- the tail spike **gp9** (18 copies per virion, assembled as a trimer).
- the tail needle **gp26** (to initiate ejection of viral DNA inside the host). The combined action of an adhesion protein (tailspike) and a tail needle (gp26) is responsible for binding and penetration of the phage into the host cell membrane (Bhardwaj, A. et al., 2011).
- and the tail factors **gp4 and gp10** (Tang, L. et al., 2005).

The protein components are organized with a combination of 6-fold (gp10, trimers of gp9), and 3-fold (gp26, gp9) symmetry (Lander, G.C. et al., 2009).

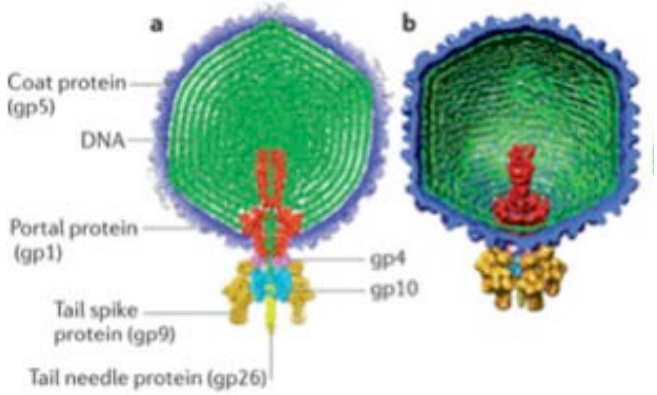


Figure 7 - P22 virion (Casjens, S.R. 2011).

P22 Assembly

One of the common features in the morphogenesis of dsDNA bacterial viruses such as λ, T4 and P22 and also of animal viruses such as the herpesviruses (O'Callaghan, D.J. et al., 1977; Rixon, F.J., 1993) and adenoviruses (D'Halluin, J.C.M. et al., 1978; Morin, N. and Boulanger, P., 1984) is that the initial product of the viral assembly pathway is not an infectious virion but a closed shell that does not contain DNA. This preformed precursor capsid is known as procapsid (Casjens, S. and Hendrix, R., 1988; Venkataram Prasad, B.V. et al., 1993), which serves as DNA packaging machine (Teschke, C.M., 2003). These precursor shells, or procapsids, include proteins termed "scaffolding proteins", not found in the mature virion, but essential for their production.

The assembly of bacteriophage P22 consists of two independent, linear pathways: the assembly of the capsid and the assembly of the tail. These two subassemblies then associate to form the infectious virion (Prevelige Jr, P. E., 2006).

The morphogenetic pathway in the bacteriophage P22 has been well characterized genetically and biochemically:

- 420 coat protein (gene *5*-encoded protein, gp5) subunits coassemble with 300 molecules of the scaffolding protein (gene *8*-encoded protein, gp8) to form the procapsid (a double-shelled structure with the outer shell of the coat protein and an inner shell of the scaffolding protein) (King, J. et al., 1973). The scaffolding proteins will not found in the mature virion but are essential for assembly (Thuman-Commike, P.A. et al., 2000; King, J and Casjens, S, 1974; Thuman-Commike, P.A. et al., 1996).
- The portal vertex, composed of a dodecamer of portal protein (gene *1*-encoded protein, gp1) and the pilot proteins (encoded by the genes *7*, *16* and *20*) are incorporated at this point (Prevelige, P. E. et al., 1988; Botstein, D. et al., 1973; King, J. and Casjens, S., 1974). The portal complex is located at one of the vertices of the icosahedral coat protein shell and is the site of both DNA entry during maturation and DNA exit during infection (Thuman-Commike, P.A. et al., 1996).
- The genomic DNA, replicated as a concatamer, enters into the P22 procapsid through the portal vertex (Tye, B. et al., 1974; Casjens, S. and Hendrix, R., 1988).
- The procapsid encapsulates and condenses the viral chromosome (King, J. et al., 1976; Bazinet, C. and King, J., 1985; Mindich, L., 2004; Mindich, L, et al. 1982; Mettenleiter, T.C. et al., 2006; King, J. et al., 1976; King, J. et al., 1973; Casjens, S., 1989). The phage-encoded protein products of genes *2* and *3* recognize a specific site on the replicated DNA and initiate packaging (Botstein, D. et al., 1973; Casjens, S. and Hendrix, R., 1988).
- The process of DNA packaging results in:
    . the exit of scaffolding subunits from the procapsid, to be recycled in subsequent rounds of procapsid assembly (King, J. and Casjens, S., 1974) and
    . expansion of the icosahedral capsid lattice (Earnshaw, W. et al., 1976).
- The portal vertex is closed by the binding of the phage encoded protein products of genes *4*, *10* and *26*.
- Tail binding (gene *9*-encoded protein, gp9 called Tailspike protein, which are the cell recognition and attachment proteins) represents the final step in the assembly pathway (Teschke, C et al., 2003).

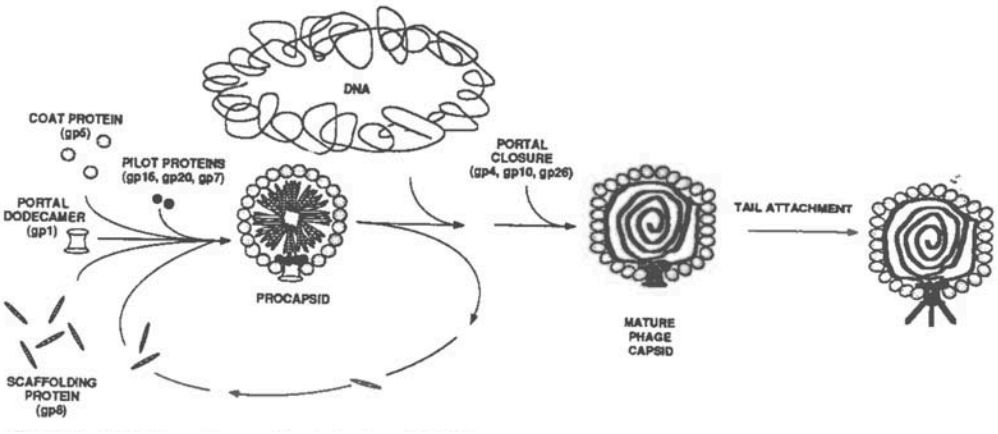


Fig 8 - Assembly pathway of phage P22 (Venkataram Prasad, B.V. et al., 1993).

1.3.2. TSP of P22

The Tailspike Protein (TSP) of phage P22 is an homotrimeric protein of 666 amino acid residues, 6 copies of which are non-covalently attached to the capsid to form the short and non-contractile tail (Israel, J. V., et al., 1967; Sauer, R.T. et al., 1982; Steinbacher, S. et al., 1994; Steinbacher, S. et al., 1996; Baxa, U. et al., 1996; Iwashita, S. and Kanegasaki, S., 1976; Iwashita, S. and Kanegasaki, S. 1973).

TSP is essential for the infection of *Salmonella* by phage P22 (Israel, J.V. et al., 1967; Botstein, D. et al., 1973):
- It's responsible for the recognition of the O-antigenic repeating units of the cell surface lipopolysaccharide (LPS) (Iwashita, S. and Kanegasaki, S. 1973; Steinbacher, S. et al., 1996),
- it displays endorhamnosidase enzymatic activity, responsible for degradation of the *Salmonella* LPS (Iwashita, S. and Kanegasaki, S., 1976). The endorhamnosidase activity is required for infection after receptor recognition, because allow a proper positioning of the phage on the cell wall surface and
- it is also involved in triggering DNA injection. Initial binding is followed by the interaction with a second receptor and the subsequent ejection of the ejection proteins, whose activity is required to active DNA to enter the cell (Israel, V., 1977; Israel, V., 1976).

TSP is present during the entire assembly period in infected cells, but the addition of the tail protein to heads is the last step in P22 morphogenesis, when fully completed heads have packaged the phage chromosome (King, J. et al, 1973; Sauer, R.T., 1982).

So P22 tail protein participates in four distinct interactions (Sauer, R.T., 1982):

1. trimer formation,
2. assembly onto phage heads,
3. binding to susceptible cells and
4. cleavage of specific sugar linkages.

This tail polypeptide is encoded by gene *9* (gp9) of P22 (Sauer, R.T. et al., 1982). The molecule of TSP is 133 Å in length and between 35 and 80 Å in diameter. Each monomer has the overall shape of a fish and is composed of six segments corresponding to the main body, the mouth, the dorsal fin, and the first, second and third segments of the caudal fin, respectively.
The amino terminal domains are on top, pointing to the phage head, and the carboxy terminal domain (amino acids 109 to 666) are on the bottom (Steinbacher, S. et al., 1996):

- Its amino terminal domain connects the TSP to the phage neck (Steinbacher, S. et al., 1997).
- Its carboxy terminal domain is responsible for the hydrolysis of the oligosaccharide receptor at the outer cell membrane (Iwashita, S. and Kanegasaki, S., 1976). This is performed through the endorhamnosidase activity of TSP, which is located around aa 500

(Schwarz, J.J. and Berget, P.B., 1989), allowing the phage particles to positionate for DNA injection (Steinbacher, S., et al., 1996).

On the other hand, the carboxy terminus is not directly involved in the assembly with heads but in trimer formation (Friget, B. et al., 1990) through the interdigitation of monomers during morphogenesis of tails (Steinbacher, S. et al., 1994).

The secondary structure is dominated by three parallel and two antiparallel β sheets. In addition, the dorsal fin contains a strongly twisted antiparallel β sheet. There are only five short α helices, α1 to α5. No disulfide bridges are present in the structure.

The main body of each subunit of the homotrimer is formed by a large parallel β helix, which permits strong intersubunit contacts (Steinbacher, S. et al., 1994).

In contrast to the main body, where the subunits form independently folded domains, the three polypeptide chains merge into a single common domain in the caudal fin, which is composed of three segments. The interdigitation of the polypeptide chains at the carboxyl termini is important to protrimer formation in the folding pathway and to thermostability of the mature protein (Steinbacher, S. et al., 1994; Steinbacher, S. et a., 1997).

The binding site is located in the central part of the β-helix, where a long, richly structured cleft is formed by a 60-residue insertion on one side and three smaller insertions of 5-25 residues on the other side. The cleft is between approximately 80 and 100 Å apart from the C terminus the protein, which is most distant from the phage head.

The folded trimer is unusually thermostable (requiring temperatures above 80ºC for irreversible inactivation) (Goldenberg, D.P. and King, J., 1981), resistant to proteolytic attack and to SDS-mediated denaturation, whereas the folding intermediates are extremely thermolabile (Danner, M. and Seckler, R., 1993).

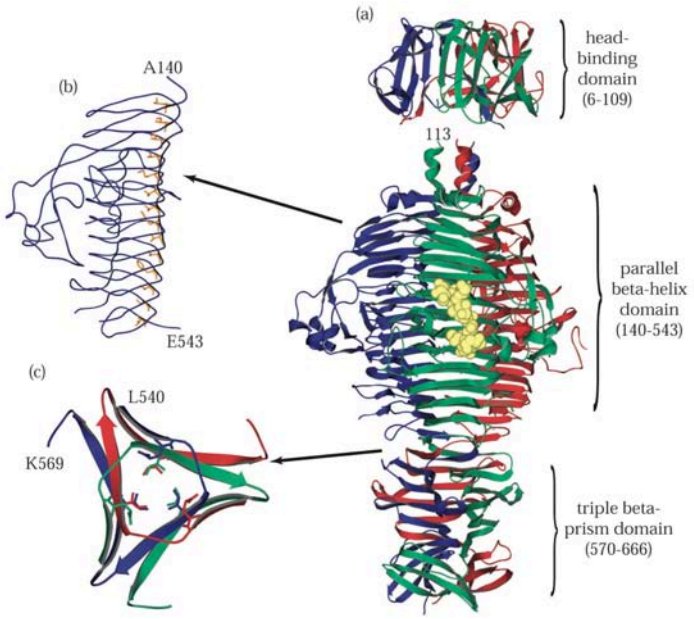


Figure 9 - P22 tailspike protein. (a) The entire P22 tailspike protein, shown bound to the nonasaccharide from S. enterica serovar 253Ty O-antigen (in yellow space-filling representation). The three subunit chains are shown in red, green, and blue. (b) An interior hydrophobic stack from one of the three identical single-chain, parallel β-helices is shown with side chains highlighted in yellow. (c) Residues 540 to 569, viewed from above and showing inwardly pointing hydrophobic residues. This region, which spans the interdigitated domain, forms one turn of a triple-stranded β-helix and is involved in trimer stability (Weigele, P.R. et al., 2003).

1.4. PEPTIDE DISPLAY & IMMUNOBIOSENSORS

1.4.1. Applications of Phages

Some of the applications of bacteriophages are the following:

Phage Therapy

Soon after phages' discovery, d'Hérelle observed increasing titers of phages during the course of recovery from dysentery and typhoid, so he concluded that the gradual adaptation of lytic phage to specific pathogens, their subsequent multiplication, and lysis of the pathogen was the mechanism of recovery. This ecological concept of phage and disease supported the effort to employ phages as therapeutic and prophylactic agents in a wide variety of infectious diseases. This clinical approach was commonly called Phage Therapy (Summers, W.C., 2001; Sulakvelidze, A. and Kutter, E., 2005; Sulakvelidze, A. et al., 2001; Kutter, E. and Sulakvelidze, A., 2004). Lytic phages are the only useful type for phage therapy, because they kill their target host cells rapidly and increase their numbers rapidly (Orlova, E.V., 2012).

Phage Therapy was administered in Eastern Europe in the 1930s (Alisky, J. et al., 1998; Sulakvelidze, A. and Kutter, E., 2005; Straub, M.E. and Applebaum, M., 1933), but it was rejected in the Western countries due due to several problems with some of these commercial phage preparations, (Straub, M.E. and Applebaum, M., 1933; Evans, A.C., 1933), badly designed clinical trials (Alisky, J. et al., 1998; Sulakvelidze, A. and Kutter, E., 2005; Sulakvelidze, A. et al., 2001; Summers W.C. 2012; Almeida, A., et al., 2009; Eaton, M.D. and Bayne-Jones, S., 1934) and due to the discovery of antibiotics.

Currently, the resistance to antibiotics due to antibiotic overuse could suggest a new interest in the possible use of phages for treat bacterial infections (Blair, J.M.A. et al., 2015; Fowler et al., 2014).

Base to study concepts in biology and virology

In the 1930s through 1950s phage research led to the discovery of a large number of key concepts in biology and virology, such as the Hershey and Chase experiments (Hershey, A. and Chase, M., 1952).

To control bacterial pathogens

As natural enemy of bacteria, phages are very useful for biological control of bacterial contamination of foodstuffs in alimentary industry, agriculture to control bacterial pathogens, such as Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas*, *Listeria*, *Salmonella*, *E. coli*, *Campylobacter*, etc.

Pollution Indicators

Three main groups of bacteriophages infecting enteric bacteria have received the greatest amount of study in the assessment of water quality: somatic coliphages, the F-specific RNA coliphages and the bacteriophages infecting *Bacteroides fragilis* (Gerba, Ch.P., 2006).

Bacterial Pathogenesis

Since phages turn some harmless bacteria into agents of disease (phage-encoded toxins of *Corynebacterium diphtheriae* (diphtheria) (Freeman, V., 1951; Mokrousov I., 2009), *Vibrio cholerae* (cholera) (Charles, R.C. and Ryan, E.T, 2011; Faruque, S.M. et al., 2000), *Clostridium botulinum* (botulism), *Streptococcus pyogenes* (scarlet fever), *Staphylococcus aureus* (food poisoning) (Sumby, P. and Waldor, M.K., 2003) and *E. coli* (Shiga toxin) (Wagner, P.L. et al., 2001)), understanding new ways in which phages contribute to bacterial pathogenesis could suggest novel strategies for the prevention and treatment of bacterial infections (Wagner, P.L. and Waldor, M.K., 2006).

Phage-Based Expression Systems

Phage, and plasmid, derivatives that had 'picked up' genes from the *E. coli* chromosome led Campbell (Campbell, A., 1962) to propose his model based on recombination between circular genomes. According to Campbell's model, segments of bacterial DNA were added to a phage genome if excision of the prophage was by an "aberrant" recombination event. These unexpected, or *illegitimate* events (Weisberg, R.A. and Adhya, S., 1977) fortuitously created the early recombinant clones that became tools at the cutting edge of research in the pioneering days of molecular biology (Müller-Hill, B. et al., 1968; Müller-Hill, B., 1975; Franklin, N. C., 1974; Cohen, S.N. et al., 1973; Rambach, A. and Tiollais, P., 1974; Murray, N.E., 2006).

Diagnostic Systems: Phage Typing

The most common use of bacteriophage in detection methodology is phage typing, used for the identification of pathogenic bacteria due to their narrow host range (Rees, C., 2006).

Some phage-based detection tests have been succesfully developed, such as:

- Reporter Phage (Ulitzur, S. and Kuhn, J., 1989; Rees, C., 2006),
- Phage Amplification Assay (Stewart, G.S.A.B. et al., 1992; Stewart, G.S.A.B et al., 1996; Stewart, G.S. et al., 1998; Mole, R.J and Maskell, T.W.O'C., 2001),
- Antibiotic Sensitivity Testing (Carriere, C. et al., 1997; Albert, H. et al., 2001; McNerney, R. et al., 2000,
- Phage-Mediated Release of ATP (Stanley, P.E., 1989; Corbitt, A.J. et al., 2000; Blasco, R. et al., 1998),
- Dual Phage Technology

Phage Display

Phage display is a process based on fusing the gene encoding a product of interest to a viral gene, which encode viral coat proteins. In this manner, the product protein will be displayed as an exterior fusion to a surface protein of the phage and its gene will be packed in the phage particle (Uhlén, M. et al., 1992). The peptide or protein sequence can be deduced from its encoding DNA sequence that resides in the phage particle or in a transductant. Amplification of the DNA of interest can take place by phage/transductant propagation or by polymerase chain reaction (PCR). By producing large populations of phage particles, each expressing a unique peptide or protein, peptide/protein libraries can be obtained. Peptides or proteins, interacting with defined molecular targets can be isolated from such libraries by enrichment through repeated cycles of panning. Hence, phage display can be thought of as a ‘‘search engine’’ of protein-target interactions (Lindqvist, B.H., 2006). The pioneering work of Smith (Smith, G.P., 1985) first demonstrated surface display of peptides in filamentous phage fd. This innovation was extended to peptide libraries of fd and M13 (Scott, J.K. and Smith, G.P., 1990; Smith, G.P. and Scott, J.K., 1993; Smith, G.P., 1993) and phagemid display was introduced (Bass, S. et al., 1990). The display of proteins such as antibody domains and combinatorial antibody libraries soon followed (McCafferty, J. et al., 1990).

Some applications of this technology are:

- the identification of peptide or protein interactions with simple organic compounds, antibodies, receptors, etc (Barbas, C.F. et al., 2011).
- as a useful tool in protein engineering and directed evolution (Hoes, R.H., 2001; Legendre, D. et al., 1999; Houshmand, H. et al., 1999).
- applications in the large sector of phage antibody display (Krebs, B. et al., 2001)
- the use of recombinant bacteriophage displaying antigens from infectious disease agents as candidate vaccines (Sloud, M. et al., 2000), to confer immune responses against the encoded peptides or proteins (Cortese, R. et al., 1994; Sioud, M. et al., 2000).
- furthermore, complex targets such as cells (Poul, M.A. and Marks, J.D., 1999) and whole tissues/organs (Pasqualini, R., and Ruoslahti, E., 1996) have been subjected to phage display analysis, exploring novel approaches for *in vivo* homing in gene/drug delivery (Monaci, P. and et al., 2001), cancer surveillance/treatment (Ruoslahti, E., 2000) and imaging.

To extend the powers of filamentous phage display to other phage systems, phages λ (Sternberg, N. and Hoess, R.H., 1995), T4 (Mullaney, J.M. and Black, L.W., 1998), T7 (Rosenberg, A. et al., 1996) and P4 (Lindqvist, B.H and Naderi, S., 1995) have also been used for peptide display.

1.4.2. Peptide Display

In order to develop recombinant antigens and vaccine components, many biological systems have been explored as carriers for display of foreign peptides. The objective is to be success reproducing features of the natural peptide, usually antigenicity and/or immunogenicity, in a solvent-exposed surface of a recombinant microorganism, virus or protein, which is suitable to be produced under laboratory conditions in high yields.

Some examples of such biological systems are the following:
- PhoE (Agterberg, M. et al., 1987) and LamB (Charbit, A. et al., 1987) *E. coli* surface proteins,
- flagellin of *S. typhimurium* (Newton, S.M.C. et al., 1989),
- several animal viruses such as poliovirus (Rose, C.S.P. and Evans, D.J., 1991) and vaccinia (Smith, G.L. et al., 1983),
- well-characterized proteins like hepatitis B core (Clarke, B.E. et al., 1987) and
- filamentous bacteriophages (Greenwood, J. et al. 1991; Di Marzo Veronese, F. et al., 1994).

With the aim to develop new vaccine components, one of the research areas of the Nanobiotechnology Laboratory of Prof. Villaverde was to design and produce multifunctional recombinant proteins, specifically focusing into the β-galactosidase and TSP, to find permissive regions where to insert peptides with biological activity, such as the Site A of FMDV.

1.4.2.1. Site A of Foot-and-Mouth Disease Virus (FMDV)

Foot-and-mouth disease is one of the economically most important diseases of farm animals (Pereira, H.G., 1981; Domingo, E. et al., 1990). It's highly contagious and infection results in the appearance of lesions in the mouth and on the feet, fever, anorexia, depression, and a fall in meat and milk production. Mortality is low, but of greater consequence to farmers is the loss in productivity and indirect losses caused by the interruption of trading in meat and dairy products. The disease can be controlled by the slaughter of affected animals or by regular vaccination with inactivated virus vaccines in enzootic areas. The principal difficulties of vaccine formulations are:

- the vaccine is made by innactivating "live" virus, so the occasional incomplete chemical inactivation of the virus could be a problem.
- FMDV comes in a number of strains or serotypes (A, O, C, Asia1, SAT1, SAT2 and SAT3), which complicates the maintenance of vaccine stocks.
- stability at refrigeration temperatures is required, which is often difficult under field conditions.

Synthetic vaccines would overcome most of these problems, but a better understanding of the antigenic determinants is required (Laver, W.G., 1990).
Foot-and-mouth disease is caused by the Foot-and-Mouth Disease virus (FMDV).
FMDV is a single-stranded, positive-sense, highly variable and small RNA virus (Cooper, P.D. et al., 1978), belonging to the Picornaviridae family. The capsid is composed of four proteins, VP1-VP4, 60 copies of each being present in the intact virion. The proteins VP1-VP3 are partly exposed on the capsid surface and VP4 is internal and much smaller (Han, S.C. et al., 2015).

In the VP1 protein the G-H loop (residues 134-160) (Hewat, E.A. et al., 1997) appears as a disordered, highly mobile protrusion of the protein exposed on the virion surface (Acharya, R. et al., 1989; Lea, S. et al., 1994; Harrison, S.C., 1989). The G-H loop protrudes from the outer capsid surface around the five-fold axis of the icosahedral structure (Acharya, R. et al., 1989; Lea, S. et al., 1994) and it has additional unique properties:

- Peptide Vaccines: The G-H loop contains the site A.

The site A is a small peptide of VP1 protein of about 15 amino acids in length and one of the major antigenic determinants of FMDV (Mateu, M.G. et al., 1995b).

The key requirement to the success of a given peptide in eliciting a neutralizing antibody response against an intact antigen is that the peptide can mimic a sufficiently large area on the surface of the virus to form a good antibody combining site. The crystallographic result for FMDV provides a satisfying explanation for the activity of the G-H loop. FMDV presents an unusual structure, a small continuous portion of VP1 appearing to behave largely independently of the rest of the virus and, since it is exposed to an extreme extent, being highly immunodominant. In serotype C the site A comprises amino acids 138 to 150 and includes several continuous, overlapping, B-cell epitopes (Mateu, M.G. et al., 1995; Strohmaier, K. et al., 1982; Acharya, R. et al., 1989; Parry, N. et al., 1990; Bittle, J.L. et al., 1982; Pfaff, E. et al., 1982). This capsid segment, either as a peptide or as part of fusion proteins, has been incorporated in a number of synthetic vaccine formulations against FMD (Verdaguer, N. et al., 1995).

- Cell Attachment Site: Furthermore, the G-H loop of VP1 includes the conserved arginine-glycine-aspartic acid (RGD) motif involved in cell recognition and attachment (Fox, G. et al., 1989; Mason, P.W. et al., 1994) since:

- all antibodies which are known to bind to the FMDV loop region prevent attachment.
- proteolytic cleavage of this loop or the C-terminal region of VP1 abolishes cell attachment (Wild, T.F. et al., 1969; Cavanagh, D. et al., 1977).
- Short peptides including the highly conserved sequence Arg-Gly-Asp at residues 145-147 inhibit virus attachment to susceptible cells (Fox, G. et al., 1989).
- The evolutionary conserved RGD motif is a well-known integrin-binding ligand (Ruoslahti, E. and Pierschbacher, M.D., 1987; Wang, G. et al., 2015). The RGD motif interacts with a vitronectin cell receptor (integrin $\alpha_V\beta_3$) in surface of mammalian cells (Berinstein, A. et al., 1995; Jackson, T. et al., 1997). RGD-containing peptides also promote internalization of different natural and recombinant viruses (Hart, S.L. et al., 1994; Wickham, T.J. et al., 1993).
- In FMDV, the RGD motif has been proposed to be the unique cell attachment site on the virus surface, because its absence abolishes infectivity (McKenna, T.S.C. et al., 1995).

60 copies of the triplet RGD are symmetrically displayed at the FMDV capsid surface around the five-fold axis. A structure for the exposed G-H loop was elucidated upon chemical reduction of a serotype O virion (Logan, D. et al., 1993). A similar structure has been determined for the G-H loop of a serotype C (isolate C-S8c1) virus, as reproduced by a 15-mer peptide (A15) complexed with the Fab fragment on an antivirus neutralizing antibody (Verdaguer, N. et al., 1995).

In both structures, the RGD motif adopts a very similar conformation (showing a helical conformation of the eight residues at the carboxy side of the RGD motif), which resembles those found in other integrin ligands. The RGD triplet also participates in the contacts with anti-site A antibodies (Verdaguer et al., 1996; Verdaguer et al., 1998), probably being relevant for the overall structure of the antigenic determinant.

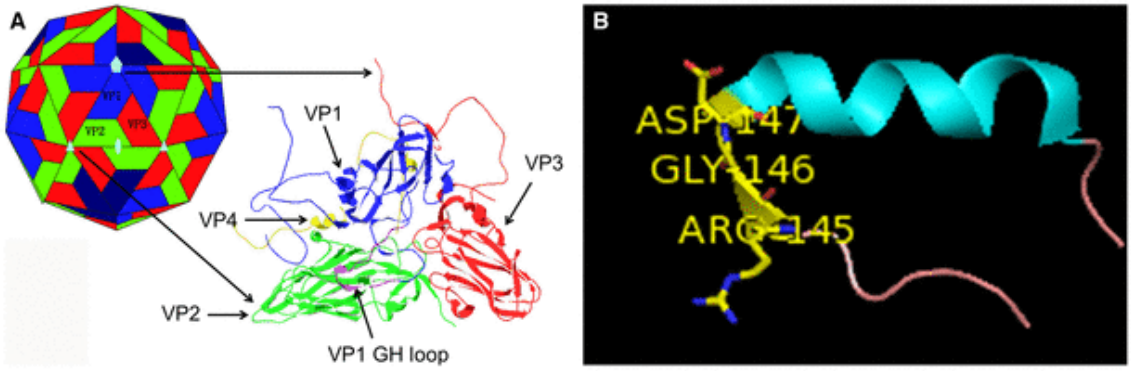


Figure 10 - (A) Schematic depiction of the viral icosahedral capsid of FMDV consisting of 60 copies each of four structural proteins (VP1-4) (VP1, blue; VP2, green; VP3, red; VP4, yellow). (B). Cartoon diagram of the VP1 G-H loop of FMDV. The conserved RGD motif is labeled and shown as sticks (Han, S.C. et al., 2015).

1.4.2.2. β-Gal Recombinant Proteins

The β-galactosidase enzyme (EC 3.2.1.23), coded by the *lacZ* gene of *E. coli*, is a high molecular mass tetrameric enzyme of four non-covalently linked subunits, each one consisting of 1023 amino acids (Kalnins, A. et al., 1983). β-galactosidase hydrolyses lactose into glucose and galactose, allowing bacteria to grow in the presence of lactose as a carbon source (Jacob, F. and Monod, J., 1961). Because it also hydrolyzes other substrates that are converted in colored compounds, this enzyme has been widely used as a molecular marker (Silhavy, T.J. and Beckwith, J., 1985). By using this property, its enzymatic activity can be easily detected in individual colonies (blue color in Lac^+ colonies growing on plates in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sambrook, J. et al., 1989) or quantified spectrophotometrically at 420 nm (yellow color in permeabilized cultures after addition of 2-nitrophenyl- β-D-galactopyranoside, ONPG) (Miller, J.H., 1972). It has been proposed the use of β-galactosidase tag in on-line monitoring production of fusion proteins and gene expression in *E. coli* (Benito, A. et al., 1993). The resolution of the crystal structure of β-galactosidase (Jacobson, R.H. et al., 1994) gave a rationale for permissiveness for insertion into some regions of the protein.

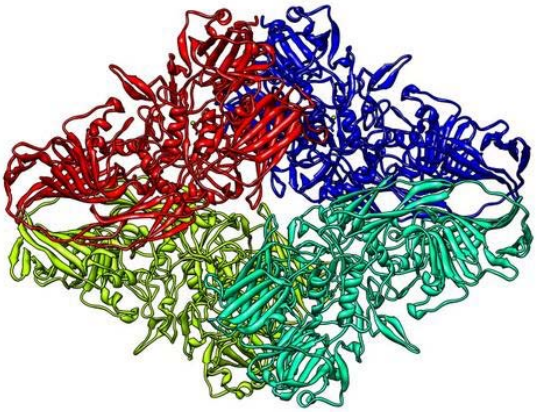


Figure 11 - Three-dimensional structure of β-galactosidase from E. coli (Jacobson, R.H. et al., 1994).

Some studies have shown that β-galactosidase can support small insertions without leading to complete enzymatic inactivation. Breul et al., randomly introduced octameric oligonucleotides in the

lacZ gene in order to corroborate the domain structure in the monomer (Breul, A. et al., 1991). Baum et al., inserted decapeptides corresponding to HIV and polio protease cleavage sites to assay viral protease activities (Baum, E.Z. et al., 1990).

Villaverde et al., searched for regions of β-galactosidase, predicted to be exposed at the molecule surface, which could accept larger insertions maintaining at least some of the enzymatic activity. They inserted a FMDV peptide of 27 amino acids (reproducing the hypervariable G-H loop sequence of VP1 capsid protein of serotype C in different solvent-exposed regions of β-galactosidase. They identified several permissive regions of β-galactosidase, for which the resulting chimeric enzymes were soluble, stable, produced in high yields and enzimatically active (Benito, A. and Villaverde, A., 1994; Feliu, J.X. and Villaverde, A., 1998; Corchero, J.L. et al., 1996).

Villaverde, A. et al. demonstrated that the FMDV RGD motif inserted in a recombinant protein is a potent ligand to promote cell attachment to susceptible cells mainly through the vitronectin receptor (Villaverde, A., et al., 1996). Villaverde, A. et al. showed that this viral peptide inserted into the β-galactosidase can direct cell targeting and delivery of the recombinant, enzymatically active β-galactosidase into cultured mammalian cells (Villaverde, A. et al., 1998). Arís, A. and Villaverde, A. characterised an RGD-tagged, cell-targeted multifunctional β-galactosidase carrying a poly-lysine-based DNA-binding domain to be explored as a suitable cell-binding tag in nonviral recombinant vehicles for targeted gene delivery (Arís, A. and Villaverde, A., 2000). They shown steady levels of gene expression for more than 3 days after transfection, representing between 20 and 40 % of those achieved with untargeted, lipid-based DNA-condensing agents. They concluded that the principle to include viral motifs for cell infection in single polypeptide recombinant proteins represents a promising approach towards the design of non-viral modular DNA transfer vectors for cell targeting and gene therapy (Arís, A. et al., 2000). A molecular model of an inserted stretch reveals a highest flexibility of the RGD tripeptide segment compared with the flanking sequences that could allow a proper accomodation to integrin receptors even in poorly antigenic conformations (Feliu, J.X. et al., 1998).

Benito, A. et al., (Benito, A. et al., 1995) studied the antigenicity of several recombinant β-galactosidases displaying the site A in different surface regions of the enzyme and they showed that in some of them, the inserted stretch mimics better than free the antigenicity of site A in the intact virus (Benito, A. et al., 1995). In particular, an insertion within an exposed loop involved in the activating interface of β-galactosidase led to a significant improvement of the overall reactivity. They concluded that the activating interface of β-galactosidase could be an adequate place for the presentation of foreign antigens. Additionally they propose that exploring recombinant proteins (β-galactosidade or others) for the display of foreign epitopes in particular sites could result in improved epitope performance and allow the design of new, more powerful antigens (Benito, A. et al., 1995).

On the other hand, Benito, A. et al. demonstrated that in the recombinant β-galactosidase M278VP1, where the Site A was inserted in a large and exposed loop of the β-galactosidase involved in the formation of the activating interface, the binding of the specific antibodies directed to the foreign peptide causes an increase of the β-galactosidase activity up to about 200% (Benito, A. et al., 1996). This insertion site seems to be sensitive enough to enzymatic modulation mediated by antibody binding. This finding could represent a new potential application of chimeric β-galactosidases as molecular sensors to detect antibodies directed against the heterologous region (Benito, A. et al., 1996).

1.4.2.3. TSP Recombinant Proteins

Carbonell, X. and Villaverde, A. investigated the TSP of P22 as a carrier for multimeric display of foreign antigenic peptides (Carbonell, X. and Villaverde, A., 1996). They showed that the carboxy terminal end of TSP can tolerate the insertion of a long peptide reproducing the site A of FMDV and that the resulting chimeric protein TSPA maintains all its biological properties, is not toxic for the host cells and can be easily produced and purified. Also that the foreign peptide is solvent exposed, accessible to antibodies and highly antigenic.

Taking advantage that the assembly reaction of the TSP to the virus particle can be also done *in vitro* at a high efficiency (Israel, J.V. et al., 1967), Carbonell, X. and Villaverde, A. explored the ability of TSPA proteins to reconstitute infectious viruses by *in vitro* assembling with tailles particles. They showed that the extracts of *E. coli* cells producing TSPA are efficient in conferring infectivity to tailless heads like the native TSP. Also they showed that mAb 3E5 is also able to neutralize P22 infectious particles containing TSPA, demonstrating a tight interaction between the antibody and the foreign peptide.

Additionally they analized the flexibility of randomly selected inner sites and both amino and carboxy termini of TSP, to accommodate foreign peptides for phage display (Carbonell, X. and Villaverde, A., 1998a). They showed that in the examined inner sites, TSP is extremely sensitive to minor sequence modifications, the folding intermediates being rapidly degraded. However, both the amino and carboxy termini are tolerant to peptide fusions, rendering stable and functional chimeric proteins. Surprisingly, the amino terminus, which connects the tail to the neck structure, can accept large peptide fusions, and the foreign amino acid stretches are solvent-exposed and highly antigenic on assembled, infectious virus particles. At difference of TSPA, the chimeric phages with ATSP are not neutralized by targeting antibodies. This finding could be of great relevance for further exploitation of TSP amino terminus for phage display purposes, since it can allow repeated round of biopanning amplification without risk of phage inactivation.

The analysis of the thermal resistance and unfolding pathway of two mutant, functional TSPs carrying end-terminal peptide fusions showed that the C-terminal fusion has minor effects on the TSP stability, but that the inserted peptide at the amino terminus significantly increases the thermal stability of TSP (Carbonell, X. and Villaverde, A., 1998b).

Carbonell, X. et al. explored the antigenic profile of the FMDV G-H loop when displayed at the C-terminus of the TSP and compared this pattern with that of the same peptide presented on different regions of the β-galactosidase surface. They showed that the chimeric TSP is highly antigenic and the viral peptide shows an antigenic profile similar to that observed on the virus surface, but especially to one among the chimeric β-galactosidases. This converging antigenic profile indicates that more than one particular framework can provide the structural requirements for an improved antigenic mimicry of a peptide inserted in a recombinant, carrier protein (Carbonell, X. et al., 1996). Additionally, Carbonell, X. et al. performed an exhaustive antigenic analysis of the same peptide displayed on 20 structurally distinct frameworks and they showed that recombinant proteins exhibit a distinguishable antigenic structure as measured by the immunoreactivity of six embraced B-cell epitopes. The observed display-induced antigenic variation prompts a careful consideration of the molecular context when evaluating output amino acid sequences from screening of peptide libraries or application of directed molecular evolution technologies (Carbonell, X. et al., 1998). Following these results, Carbonell, X. and Villaverde, A. proposed that TSP and P22 could be a new vehicle for presentation of antigenic recombinant peptide.

1.4.3. Immunobiosensors

Biosensors are molecular sensors that combine a biological recognition mechanism with a physical transduction technique (Cornell, B.A. et al., 1997).
The development of most biosensors involves:

1. the identification of a naturally occurring macromolecule that provides the desired analyte specificity (typically an enzyme or antibody),
2. the discovery of a suitable signal and
3. the construction of a detector adapted to the macromolecule in question (Hellinga, H.W. and Marvin, J.S., 1998).

Immunosensors are affinity ligand-based biosensor in which the immunochemical reaction is coupled to a transducer. The fundamental basis of all immunosensors is the specificity of the molecular recognition of antigens by antibodies to form a stable complex (Luppa, P.B. et al., 2001).

Two strategies have been used to design modular protein-engineering systems for biosensor development:
1. To find a protein with the appropriate specificity and introduce a signal-transduction function such as a flurophore (Giuliano, K.A. and Taylor, D.L., 1998):
1.1. Bacterial ligand-binding proteins, where binding is accompanied by a large conformational change:

- 1.1.1. phosphate binding protein (PBP) (Brune, M. et al., 1994),
- 1.1.2. maltose binding protein (MBP) (Marvin, J.S. et al., 1997) and
- 1.1.3. glucose/galactose binding protein (GBP).

1.2. Metalloproteins, such as Zinc fingers or Carbonic Anhidrase.

2. To identify a protein with a particularly well-behaved intrinsic signal-transduction function and construct appropriate binding sites.

2.1. Enzymes that have been engineered to display foreign antigenic peptides, becoming enzymatically responsive to binding of anti-peptide antibodies by either an increase or decrease of the enzymatic activity:

2.1.1. β-galactosidase

It has been shown that the binding of specific antibodies directed to the Site A inserted in a large and exposed loop of the β-galactosidase (M278VP1) causes an increase of the β-galactosidase activity up to about 200% (Benito, A. et al., 1996).

This insertion site seems to be sensitive enough to enzymatic modulation mediated by antibody binding.

2.1.2. Alkaline phosphatase

The *E. coli* alkaline phosphatase has been engineered, inserting antibody-binding epitopes into its active site for the detection of antibodies (Brennan, C. et al., 1994; Brennan, C.A. et al., 1995). Short (13-15 amino acids), linear, heterologous epitopes derived from the human-immunodeficiency-virus (type I) gp 120 or hepatitis-D viral proteins could be inserted at a distance of 35-40 Å from the active site. Binding of monoclonal antibodies (mAbs) resulted in significant inhibition of enzyme activity (as much as 40 %). The introduction on an additional single point mutation, known to increase the general flexibility of the active site, resulted in the activation of enzyme activity upon mAb binding (to as much as 300 %). This suggests that the modulation of the activity by the bound mAb occurs via an allosteric mechanism involving changes in conformation states, rather than through steric hindrance. The observed modulation (three- to fourfold) and probable independence of the inserted amino acid sequence suggest that this system may be generally applicable for the detection of antibodies or protein receptors.

2.1.3. β-Lactamase

It has been shown that the phage displayed TEM-1 β-lactamase has been engineered to generate enzymes that can be used in homogeneous immunoassays because their activity can be modulated by binding to monoclonal antibodies (Mabs) raised against an unrelated protein (Legendre, D. et al., 1999).

2.2. Green fluorescent protein (GFP). Since GFP contains a highly fluorescent fluorophore, this protein is the perfect candidate for constructing an optical biosensor with an intrinsic fluorescent transducer function, provided that an appropriate binding site can be introduced (Romoser, V.A. et al., 1997; Myyawaki, A. et al., 1997).

2.3. α-Haemolysin. Staphylococcal α-haemolysin is a bacterial transmembrane pore protein, which has been used to construct electrochemical sensors. In these sensors the signal-transduction mechanism is provided by the measurement of electrical currents resulting from the opening and closing of protein pores in membranes (Braha, O., et al., 1997).

2.4. Antibodies. In some cases, it has been possible to use chemical modification for the site-specific attachment of reporter groups (Pollack, S.J. et al., 1988). Also, a family of electrochemical sensors has been developed, combining antibodies and pore-forming transmembrane peptides (Cornell, B.A. et al., 1997).

2 - OBJECTIVES

OBJECTIVES

The current work has been focused on the bacteriophage P22's infection upon bacterial colonies.

- Since it has been shown the extreme adaptability of viral genomes to their continuously changing environment (Domingo, E. et al., 1985; Steinhauer, D.A. and Holland, J.J., 1987; Lwoff, A. et al., 1950; Blyth, W.A. et al., 1976) one of the objectives of this work was to analyze the viral spread strategy followed by the temperate phage P22 in aging bacterial colonies to ensure its viral maintenance when the host survival is compromised.

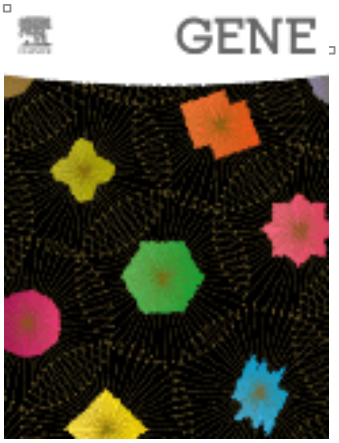
- It was proposed that TSPA and P22TSPA obtained by *in vitro* assembly of tailess P22 heads with recombinant TSPA as new vehicle for peptide display (Carbonell, X. and Villaverde, A., 1996), and on the other hand it was suggested the β-galactosidase as a potential new molecular sensor to detect antibodies directed against the heterologous region (Benito, A. et al., 1996). Then, other objectives of this work were:
  - to further analyze the reactivation mechanism of recombinant β-galactosidases.
  - to explore if P22TSPA could be used as immunobiosensor to detect antibodies directed against the heterologous region, throughout the changes of infectivity of peptide-displaying P22 upon bacterial colonies.

3 - RESULTS & PUBLICATIONS

This thesis is based on the results showed in the following publications:

PART I - Analysis of the Bacteriophage P22 Viral Spread within Bacterial Populations

PUBLICATION I



ELSEVIER

Gene 202 (1997) 147–149

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

Viral spread within ageing bacterial populations

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PUBLICATION II

ELSEVIER

FEMS Microbiology Letters 170 (1999) 313–317

FEMS
MICROBIOLOGY
LETTERS

RecA-dependent viral burst in bacterial colonies during the entry into stationary phase

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PUBLICATION III

Microbiol. Res. (2001) **156,** 35–40
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Microbiological Research
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Phage spread dynamics in clonal bacterial populations is depending on features of the founder cell

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PART II - Characterization of Peptide-Displaying P22 as Immunobiosensor

PUBLICATION IV

FEBS 21072 FEBS Letters 438 (1998) 267 271

Distinct mechanisms of antibody-mediated enzymatic reactivation in β-galactosidase molecular sensors

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Corrigendum

Corrigendum to: **Distinct mechanisms of antibody-mediated enzymatic reactivation in β-galactosidase molecular sensors**

[*FEBS Letters 438 (1998) 267–271*][1]

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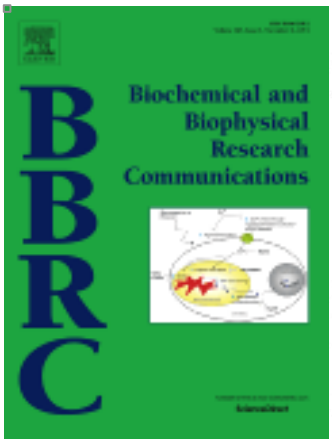
PUBLICATION V



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Detection of Molecular Interactions by Using a New Peptide-Displaying Bacteriophage Biosensor

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Received July 3, 1999

3.1. PART I - Analysis of the Bacteriophage P22 Viral Spread within Bacterial Populations

PUBLICATION I: Viral spread within ageing bacterial populations. Gene 202, 147-149

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Gene 202 (1997) 147–149

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

Viral spread within ageing bacterial populations

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Received 4 March 1997; accepted 1 August 1997; Received by M. Salas

Abstract

The viral spread within isolated host populations has been studied throughout the growth of P22-infected *Salmonella* cell colonies. By using an integration mutant of this bacteriophage, horizontal and vertical transmission have been analyzed independently. The data obtained show that both strategies are not simultaneous but consecutive during the colony development. Lytic cycles are tightly repressed during the exponential cell growth but stimulated in independent colonies with remarkable synchrony when the cell division rate decreases. The coincidence of the viral outburst and the decay of bacterial replicative fitness is a new example of the extreme viral competence in exploiting the host cells as dissemination vehicles for viral genomes. © 1997 Elsevier Science B.V.

Keywords: P22; Bacteriophage; Lysogeny; Colony growth; SOS response; (*Salmonella*)

PUBLICATION II: RecA-dependent viral burst in bacterial colonies during the entry into stationary phase. FEMS Microbiology Letters 170, 313-317

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FEMS Microbiology Letters 170 (1999) 313–317

FEMS
MICROBIOLOGY
LETTERS

RecA-dependent viral burst in bacterial colonies during the entry into stationary phase

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Abstract

We have explored the nature of the sudden viral amplification observed during the ageing of P22-infected lysogenic colonies of *Salmonella typhimurium* [Ramírez, E, and Villaverde, A. (1997) Gene 202, 147–149]. By a comparative analysis of the wild-type P22 and a P22 integration mutant, it has been shown that the conditions promoting prophage induction occur in only a

PUBLICATION III: Phage spread dynamics in clonal bacterial populations is depending on features of the founder cell. Microbiol. Res. 156, 1-6

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Phage spread dynamics in clonal bacterial populations is depending on features of the founder cell

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Abstract

Plate-cultured bacterial colonies are intriguing models to study host-parasite interactions in senescent populations. During the growth of bacteriophage-infected colonies there is a synchronous prophage induction episode among lysogenic cells that allows a dramatic but time-restricted amplification of viral genes (Taddei *et al.* 1995; Villarroya *et al.* 1998), among others. Isolated bacterial colonies offer a natural, non-homogeneous model to analyse ageing in bacterial populations. In this context, we have studied cell infection and viral spread within bacterial colonies during

3.2. PART II - Characterization of Peptide-Displaying P22 as Immunobiosensor

PUBLICATION IV: Distinct mechanisms of antibody-mediated enzymatic reactivation in β-galactosidase molecular sensors. FEBS Letters 438, 267-271

FEBS 21072

FEBS Letters 438 (1998) 267–271

Distinct mechanisms of antibody-mediated enzymatic reactivation in β-galactosidase molecular sensors

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Received 7 July 1998; received in revised form 1 October 1998

Abstract **The antibody-mediated reactivation of engineered *Escherichia coli* β-galactosidases [Benito et al. (1996) J. Biol. Chem. 271, 21251–21256] has been thoughtfully investigated in three recombinant molecular sensors. Proteins M278VP1, JX772A and JX795A display the highly antigenic G-H loop peptide segment of foot-and-mouth disease virus VP1 protein, accommodated in different solvent-exposed loops of the assembled tetramer. These chimaeric enzymes exhibit a significant increase in enzymatic activity upon binding of either monoclonal antibodies or sera directed against the inserted viral peptide. In JX772A but not in M278VP1, the Fab 3E5 antibody fragment promotes reactivation to the same extent as the**

vation is easily detectable by a rapid, standard and quantitative colorimetric assay [15]. For this reason, β-galactosidase, as previously shown for alkaline phosphatase [17], could be the basis for a new generation of enzymatic molecular sensors, that would, however, require further engineering for a more confident and sensitive use in homogeneous assays.

Since the molecular basis of antibody-mediated enzymatic reactivation had not been explored, we have analysed here both the enzymatic constants of reactivated β-galactosidase sensors and the putative requirement of bivalent antibody binding for reactivation. The data obtained prompted us to

Corrigendum to: Distinct mechanisms of antibody-mediated enzymatic reactivation in β-galactosidase molecular sensors [FEBS Letters 438 (1998) 267-271] FEBS Letters 473, 123

FEBS 23615

FEBS Letters 473 (2000) 123

Corrigendum

Corrigendum to: **Distinct mechanisms of antibody-mediated enzymatic reactivation in β-galactosidase molecular sensors**

[*FEBS Letters 438 (1998) 267–271*][1]

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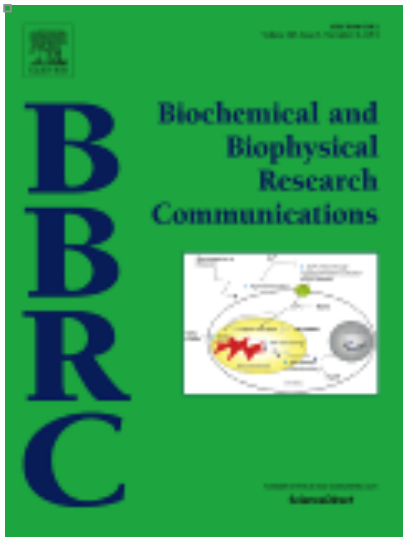
The authors had previously explored the reactivation mechanisms of three β-galactosidase-based enzymatic sensors, namely M278VP1, JX772A and JX795A. However, cross-contamination between the JX772A and JX795A protein stocks used for this purpose has since been noticed. After a carefully repeated analysis of new pure protein stocks, the authors have observed a very poor reactivation of JX772A upon antibody binding (within the experimental background) and have also determined new parameters for JX7795A, this enzyme clearly belonging to the class II sensors. These data are given in corrected Tables 1 and 2.

PUBLICATION V: Detection of Molecular Interactions by Using a New Peptide-Displaying Bacteriophage Biosensor. Biochemical and Biophysical Research Communications 262, 801-805



Biochemical and Biophysical Research Communications **262,** 801–805 (1999)
Article ID bbrc.1999.1268, available online at http://www.idealibrary.com on IDEAL®

Detection of Molecular Interactions by Using a New Peptide-Displaying Bacteriophage Biosensor

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Received July 3, 1999

Foreign peptides fused to the carboxy terminus of P22 tailspike protein are solvent-exposed and highly antigenic when displayed on the surface of infectious virus particles. Binding of an anti-peptide specific Fab antibody fragment enhances the infectivity of chi-

quired a close vicinity of the inserted epitopes to the active site. Since some phage-displayed, engineered β-lactamases show also an enhancement in their activity in response to antibody binding (5), it appears that not only free but also enzymes attached to a complex macromolecular structure such as a viral particle can

4 - DISCUSSION

4.1. PART I - Analysis of P22 Viral Spread within Bacterial Populations

Some examples of the extreme adaptability of viral genomes to their continuously changing environment are the RNA viruses and retroviruses variability (Domingo, E. et al., 1985; Steinhauer, D.A. and Holland, J.J., 1987; Barre-Sinoussi, F., 1996). This is linked to the fact that viral populations can gain replicative fitness by fixation of naturally occurring mutations that improve their biological properties in a given context, or viruses which can also stimulate their multiplication in response to conditions that, bieng adverse for the host, could cause loss of viral genetic information (such as the reactivation of proviral genomes or expression of lytic genes after damaging host DNA (Lwoff, A. et al., 1950; Roberts, J.W. and Roberts, C.W., 1975; Blyth, W.A. et al., 1976; Vogel, J. et al., 1992)).
In this work we have explored the viral spread within isolated host populations throughout the growth of P22-infected *Salmonella* cell colonies in order to analyze the adaptative responses of the virus to the ageing, and consequent loss of replicative fitness, of the host cells. By using as a model a mutant bacteriophage unable to integrate its prophage DNA into the bacterial chromosome, having then the viral genome in an episomal form that does not amplify in the absence of lytic cycles (Susskind, M. and Botstein, D., 1978), horizontal and vertical transmission have been analyzed independently, within a clonal population of infected bacterial cells. Bacterial colonies grown on solid medium have become an interesting model to study host-parasite interactions in ageing populations.

The obtained results show that both strategies are not simultaneous but consecutive during the colony development. It was observed in separated colonies a coincidence between the viral outburst and the entry into stationary phase (when the decay of bacterial replicative fitness occurs). During the exponential cell growth lytic cycles are tightly repressed, thus guaranteeing an efficient host spreading. When the growth rate of the host population decreases, lytic functions are expressed in independent colonies and there is a sudden increase of viral infectious particles in lysogenic colonies, becoming the surviving cells infected in very few generations. The observed asymmetry in the occurrence of these transmission strategies seems to tend to maintain the viability of infected cells when exponentially growing, but promotes viral production and efficient horizontal spread when entering into the stationary phase.

The basis of the proviral reactivation probably lies on the activation of the SOS system of the bacterial cells (Taddei, F. et al., 1995). In cultures of Gram-negative bacteria, the transition from the exponential growth phase to the stationary phase is accompanied by dramatic changes in the cell metabolism that allow maintenance of cell viability under nutrient starvation (Ishihama, A., 1997; Kolter, R., 1993). It has been reported that SOS DNA repair

activities are induced by DNA damage or by the arrest of intact DNA replication (Walker, G.C., 1985; Reddy, M and Gowrishankar, J., 1997), with the consequent generation of mutations (Taddei, F. et al., 1995). Upon its activation by single-stranded DNA, RecA promotes the autodigestion of LexA (Kim, B. and Little, J.W., 1993) and the lytic repressors of bacteriophages λ and P22 (Phizicky, E.M. and Roberts, J.M. 1980; Kim, B. and Little, J.W., 1993). The coincidence of the viral outburst and the decay of bacterial replicative fitness in independent colonies reflects an extreme proviral sensitivity to the host growth potential, and reveals a programmed adaptability of viral replicative fitness to better maximize the host as a dissemination vehicle for viral genomes.

Additionally a comparative analysis of prophage induction dynamics of wild-type P22 (wt P22) and a P22 integration mutant, and also the exploration of the viral amplification in a $RecA^-$ host was performed in senescent host populations. The results obtained show a higher amplification of the integration mutant, revealing that more cycles of viral multiplication were carried out by this phage. Since the P22 superinfection exclusion system does not allow further infection of lysogenic cells (Susskind, M.M. et al., 1978), in wt P22-infected cells the viral amplification can only be explained by prophage induction, but not by further cell infection mediated by the released virus particles. The increase of free viruses is only observed during the growth rate transition and an associated decrease of viable cells is not detectable. Therefore, the conditions promoting prophage induction must affect only a small fraction of the cell population and briefly only during a very restricted period of time, during the entry into the stationary phase.

On the other hand, the higher titres reached by the P22 integration mutant can be explained by subsequent infection of phage-free cells by virus particles originating from the initial prophage induction burst. The fraction of cells subsequently infected by the P22 integration mutant, which are responsible for the prolonged amplification of this virus, might be unable to support prophage induction in P22 wt-infected colonies. The differences between the wild-type phage and the P22 integration mutant suggest that the period in which the cells support multiplication of externally infecting phages could be longer than the period in which the prophage produces lytic cycles in lysogenic cells. Moreover, they also indicate the existence of at least two different cell sub-populations within ageing colonies regarding phage infection profile.

The analysis of prophage induction dynamics in a $RecA^-$ host showed a non-synchronous P22 amplification in independent colonies, being the viral amplification a random event. Previously it was shown that the cessation of DNA replication in ageing and nutritionally restricted bacterial cultures could be responsible for RecA protein activation (Taddei, F. et al., 1995; D'Ari, A.M. and Moreau, P.L., 1993). Also that the RecA protein activated by DNA damage or by the arrest of intact DNA replication (Reddy, M and Gowrishankar, J., 1997), promotes the cleavage of lambda and P22 phage repressor, resulting in prophage induction

(Walker, G.C., 1985; Phizicky, E.M. and Roberts, J.M., 1980; Kim, B. and Little, J.W., 1993). This would indicate that the sudden viral burst observed during the entry into stationary phase in separated colonies is dependent on a functional RecA protein.

In order to determine if the lytic cycles were stimulated by a decrease in the growth rate, we studied if prophage induction could be reproduced in continuous cultures by reducing the growth rate through the dilution rate. The results showed that the concentration of free particles did not increase after a dilution rate down-shift. This could imply that a simple growth rate down-shift is not sufficient to promote RecA-dependent phage multiplication. However, the carbon limiting conditions in continuous cultures may not permit viral biosynthesis. And on the other hand, the physiological heterogeneity of an ageing bacterial colony, which is not present in a homogeneous continous culture, could be a prerequisite for successful viral spread. The relative position of individual cells could make nutritional resources differently available during the transition to the stationary phase. In this period, a mild RecA activation could occur in cells still able to support viral biosynthesis, whereas a more severe starvation of the remaining population could be limiting for further viral propagation.

To better understand the complex relationship between bacteriophages and bacterial host cells in colonies, we explored the possible influence of the state of the founder, infected cell on the variability of the pre-burst time, namely the period of colony growth before free viral particles are released. To explore it, we followed an approach consisting on the systematic reculture of infected cells taken from differently aged infected colonies, and the subsequent detection of the viral burst occurrence in these secondary colonies.

The results presented in this work prove that the pre-burst time and the whole dynamics of phage spread is influenced by the history of the infected colony from which the founder cell derives. Although the nature of this founder, cell-driven "memory" of the derived clonal population remains unclear, a physiological explanation based on the heterogeneity within bacterial colonies can be suggested. A mild nutrient starvation after some hours of exponential growth would induce the SOS response by described mechanisms (Taddei, F. et al., 1995) in an increasing fraction of cell population. In this line, significant pH heterogeneity has been observed in *Salmonella* colonies (Walker, S.L. et al., 1997), indicating gradients of metabolite formation and therefore of nutrient availability. In SOS-activated cells, the RecA-mediated expression of lytic viral genes (Kim, B. and Little, J.W., 1993; Phizicky, E.M. and Roberts, J.M., 1980) would initiate an episode of viral production that would decline when starving conditions will become more severe. Therefore, aged, non-productively infected cells could be increasingly committed to support lytic cycles that would take place when nutrients become again available by re-culturing on fresh medium, after pre-burst times that become progressively shorter.

This would be in agreement with the stronger influence that t_1 (the time period when the growth of the primary colonies took place) has on viral burst occurrence during early C_1 (primary colonies) growth. From 45 h on, there is a poor dependence of the viral spread profile in derived C_2 (secondary colonies), indicating that in aged founder cells, the commitment state is more efficient and that in addition, a plateau has been reached. However, it would be difficult to assume that a mere unbalance of phage regulatory proteins of a higher titre of activated RecA protein within the founder cell could be maintained in the new colony during several cell doublings. Critical levels of key proteins would only account for immediate viral induction after re-culture.

Eventually, genetically transmissible molecular determinants, such as RecA-dependent variations of the phage genome copy number like those observed in non viral episomes (Bertrand-Burggraf, E. et al., 1989; Benito, A. et al., 1993b), could contribute to the ability of a founder, infected cell to set an average pre-burst time after a period of colony growth, and to define the complex dynamic interaction between host and viral parasite in the future colony.

4.2. PART II - Characterization of Peptide-Displaying P22 as Immunobiosensor

Villaverde, A. et al. demonstrated that:

- β-galactosidase has shown several permissive regions for inserting the G-H loop of FMDV. In these cases, the resulting chimeric enzymes are soluble, stable, produced in high yields and enzimatically active (Benito, A. and Villaverde, A., 1994; Feliu, J.X. and Villaverde, A., 1998; Corchero, J.L. et al., 1996).
- the FMDV RGD motif inserted in the β-galactosidase is a potent ligand to promote cell attachment to susceptible cells mainly through the vitronectin receptor (Villaverde, A., et al., 1996) and also it can direct cell targeting and delivery of the recombinant, enzymatically active β-galactosidase into cultured mammalian cells (Villaverde, A. et al., 1998).
- some recombinant β-galactosidases, where the Site A was inserted in a large and exposed loop (amino acids 272-287) of the enzyme involved in the activating interface:
    - the inserted peptide mimics better than free the antigenicity of site A in the intact virus (Benito, A. et al., 1995) and
    - the binding of the specific antibodies directed to the foreign peptide causes an increase of the β-galactosidase activity (protein M278VP1) up to about 200% (Benito, A. et al., 1996).

This insertion site in the protein M278VP1 seems to be sensitive enough to enzymatic modulation mediated by antibody binding, showing a titre-dependent increase in enzymatic activity upon binding to anti-VP1 specific serum. This last finding could represent a new potential application of chimeric β-galactosidases as molecular sensors to detect antibodies directed against the heterologous region (Benito, A. et al., 1996).

Binding of either sera or monoclonal antibodies elicited against the inserted peptide results in a partial recovery of the enzymatic activity that had been lost upon peptide insertion (Benito, A. and Villaverde, A., 1994). This reactivation is easily detectable by a rapid, standard and quantitative colorimetric assay (Benito, A. et al., 1996). For this reason, β-galactosidase, as previously shown for alkaline phosphatase (Brennan, C.A. et al., 1995), could be the basis for a new generation of enzymatic molecular sensors, that would, however, require further engineering for a more confident and sensitive use in homogeneous assays. Since the molecular basis of antibody-mediated enzymatic reactivation had not been explored, we have analysed both the enzymatic constants of reactivated β-galactosidase sensors and the putative requirement of bivalent antibody binding for reactivation.

To investigate if a bivalent antibody binding is required to restore the activity in M278VP1, the responses to the whole mAb 3E5 and its Fab fragment were compared. The results showed that the recombinant enzyme M278VP1 didn't respond to the 3E5 Fab fragment, indicating that bivalent interaction might be required for activation of M278VP1.

To study the mechanisms responsible for the reactivation, enzymatic properties, such as k_m and k_{cat} were analyzed in the case of the M278VP1 and in the case of other recombinant β-galactosidase, JX795A:

- In the case of the M278VP1, the enzyme-antibody complex showed a decreased k_m with an unmodified k_{cat}.
- In the case of the JX795A, the results showed an unmodified k_m with an increased k_{cat} in the enzyme-antibody complex.

These results imply that:

- Reactivation of M278VP1 (decreased k_m with an unmodified k_{cat}) observed with antibodies, but not with Fabs, might involve conformational changes in the active site that could allow higher substrate diffusion and therefore an enhanced binding. In the M278VP1 the antigenic peptide is inserted in the activating interface, which is involved in the formation of the active site. The inserted peptide could cause steric impediments to the access of substrate and the antibody binding (not the Fab binding) could reduce these steric impediments.
- Reactivation of JX795A (unmodified k_m with an increased k_{cat}) observed with immune guinea pig sera, could be due to an increase in the actual number of active molecules promoted by the antibody, which might result from tetramer stabilisation and the consequent displacement of the monomer-tetramer equilibrium.

Acoording to reactivation profiles, classes I and II have been proposed for β-galactosidase sensors:

- class I: improved substrate binding, such as observed in M278VP1.

  In M278VP1, the acceptor site is a protruding loop extending towards the active site of the partner monomer, forming the activating interface between the components of the dimer (Jacobson, R.H., 1994). It's have been proposed that this reactivation is mediated by bivalent binding to targets which are displayed in the vicinity of active sites. Reactivation must occur due to local conformational tensions generated during bivalent binding.

- class II: increase in catalysis rate, such as observed in JX795A.

  In JX795A, the receiving, solvent-exposed loop embraces the Gly^{794}, an amino acid residue involved in substrate binding (Martínez-Bilbao, M. and Huber, R.E., 1994). It's have been proposed that this reactivation could be mediated by antibody

binding to target peptides positioned far from active sites. Reactivation must occur through long-distance conformational modifications.

These results could be of great importance for the design of improved enzymatic sensors and can contribute to the general understanding of protein structure-function relationships. Following these results obtained with the β-galactosidase, and others obtained with some phage-displayed, engineered β-lactamases which also an enhancement in their activity in response to antibody binding (Legendre, D. et al., 1999), it appears that also proteins attached to a complex macromolecular structure such as a viral particle (such as TSP in the P22 phage) can be explored as immunobiosensors.

Carbonell, X. and Villaverde, A. demonstrated that the carboxy terminal end of TSP is permissive to long peptide insertions, resulting in biologically active, non-toxic chimeric proteins able to assemble with P22 heads and to promote infection of *Salmonella* host cells. Also they demonstrated that the peptides inserted in the C terminus of bacteriophage P22 TSP (TSPA) are solvent-exposed, accessible to antibodies and highly antigenic in both TSP trimers and whole bacteriophage particles (Carbonell, X. and Villaverde, A., 1996). They showed that mAb 3E5 is also able to neutralize P22 infectious particles containing TSPA, demonstrating a tight interaction between the antibody and the foreign peptide (Carbonell, X. and Villaverde, A., 1996). At difference of TSPA, the chimeric phages with ATSP are not neutralized by targeting antibodies (Carbonell, X. and Villaverde, A., 1998a). Following these results, Carbonell, X. and Villaverde, A. proposed that TSP and P22 viruses could be a new vehicle for peptide display.

We have explored the effect of different antibodies and their respective Fab fragments anti-Site A on the infectivity of chimeric P22 particles assembled *in vitro* of tailless P22 heads with recombinant TSPA (P22TSPA) (Carbonell, X. and Villaverde, A., 1996). The results showed a slight neutralising effect of mAb 3E5 on P22TSPA but not in the control wild type (wt), in agreement with previous observations (Carbonell, X. and Villaverde, A., 1998a). But it was shown that the binding of 3E5 Fab increases the titre of infectious P22TSPA particles by more than 500% and the enhancement of phage infectivity is dependent on Fab concentration. In wild type particles was observed a 2-fold increase, may be caused by a cross-reactivity of 3E5 and TSP, as previously observed (Carbonell, X. and Villaverde, A., 1998a). With the other two tested antibodies, 4C4 and SD6, no effects were observed, may be because these antibodies are distinct against different FMDV B-cell epitopes that recognised by 3E5 (Carbonell, X. et al., 1998). The observed increase of the infective titre indicates that peptide-displaying bacteriophages can act as molecular biosensors that are responsive to specific molecular interactions.

To further explore the nature of the phage activation, we have studied its dependence on the amount of TSP molecules attached to the phage heads. In complete viral particles, 6 copies of TSP are linked to the capsid neck, but particles containing 5, 4 or 3 copies of TSP are still infectious (Israel, J.V. et al., 1967). The results showed that the reactivation by 3E5 Fab increases concomitantly with the molar amount of TSP, reaching a level of 900% when an average of 10^5 copies of TSP trimer is added per head.

To study how the antibody binding could promote an enhancement in pfu concentration over the original titre obtained in antibody-free phage suspensions, a detailed analysis of phages stored for 24 h after assembling was done. The results showed a decrease of infectivity in TSPA particles that is also depending on tail protein concentration. At conditions representing a TSPA molar excess respective to phage heads, the remaining infectious particles are less than 5 %.

This tail-dependant decrease in titre is the critical factor for activation, since in the presence of antibody the concentration of infectious particles is recovered up to the same titre at different tail protein-head ratios. At ratios lower than 1, the detected pfu are mainly accounted by a low concentration of full virions accompanying the tailless heads. These particles maintain a nearly constant titre after storage, suggesting that the presence of the foreign peptide in TSPA could impair the stability observed in the *in vivo* assembled particles.

In agreement, when the *in vivo* assembling involves recombinant TSP lacking the foreign peptide, the resulting particles are more stable, although still mild inactivation is detectable. It seems the *in vitro* assembling could promote less stable head-tail interactions than *in vivo*. Alternatively, it could be due to slight differences in the end terminal amino acid sequences between recombinant and wild type TSP (additional EF peptides at the C-terminal in TSPA), resulting from the cloning strategy (Carbonell, X. and Villaverde, A., 1996), which could promote conformational defects affecting tail-spike folding and performance during infection (would be in agreement with the joining of the foreign peptide at the C-terminal has detectable influences on TSP folding pattern (Carbonell, X. and Villaverde, A., 1998b). In this way, it seems that the binding of the antibody fragment would restore only the conformational defects promoted by the FMDV peptide, recovering viral infectivity up to the titre exhibited by P22TSP phages.

A molecular model of a TSPA trimer upon interaction with Fab showed that no sterical restrictions to the binding of three antibody fragments to one TSPA trimer are predicted. The TSP active site remains far from the Fab-binding area, but the neutralising effect of the whole mAb might indicate a partial hiding of critical residues upon interaction with a bigger

molecule. By other side, it cannot be excluded that mAb 3E5 could promote mutually compensation between neutralising and reactivating effects.

Irrespective of the mechanism involved in restoring TSPA performance, we have shown that peptide-displaying bacteriophages can be further explored as immunobiosensors to detect specific molecular interactions.

5 - CONCLUSIONS

Conclusions of the Analysis of P22 Viral Spread within Bacterial Populations

1. By using an integration mutant of P22, it has been shown that P22 viral spread within bacterial populations follows two consecutive schemes. During the exponential cell growth the lytic cycles are tightly repressed, thus guaranteeing an efficient host spreading. During entry into the stationary phase, when the cell division rate decreases, the lytic functions are expressed in independent colonies with remarkable synchrony, showing a sudden increase of viral infectious particles in lysogenic colonies. The coincidence of the viral outburst and the decay of bacterial replicative fitness reveal a programmed adaptability of viral replicative fitness to better maximize the host as dissemination vehicles for viral genomes.

2. Exploring the prophage induction dynamics of wild-type P22 (wt P22) and a P22 integration mutant a higher amplification of the integration mutant was observed, revealing that more cycles of viral multiplication were carried out by this phage.

3. Since the P22 superinfection exclusion system does not allow further infection of lysogenic cells, in wt P22-infected cells the viral amplification can only be explained by prophage induction, but not by further cell infection mediated by the released virus particles. The increase of free viruses is only observed during the growth rate transition and an associated decrease of viable cells is not detectable. Therefore, the conditions promoting prophage induction must affect only a small fraction of the cell population and only during a very restricted period of time, during the entry into the stationary phase.

4. On the other hand, the differences between the wild-type phage and the P22 integration mutant suggest that the period in which the cells support multiplication of externally infecting phages could be longer than the period in which the prophage produces lytic cycles in lysogenic cells. Moreover, they also indicate the existence of at least two different cell sub-populations within ageing colonies regarding phage infection profile.

5. The analysis of prophage induction dynamics in a $RecA^-$ host showed a non-synchronous P22 amplification in independent colonies, indicating that the sudden viral burst observed during the entry into stationary phase in separated colonies is dependent on a functional RecA protein.

6. It was shown that the viral burst observed in bacterial colonies cannot be reproduced in continuous culture by a mere decrease of the growth rate. This could imply that a simple growth rate down-shift is not sufficient to promote RecA-dependent phage multiplication. However, the carbon limiting conditions in continuous cultures may not permit viral biosynthesis. And on the other hand, the physiological heterogeneity of an ageing

bacterial colony, which is not present in a homogeneous continous culture, could be a prerequisite for successful viral spread.

7. Following an approach consisting on the systematic reculture of infected cells taken from differently aged infected colonies, and the subsequent detection of the viral burst occurrence in these secondary colonies, we have shown that the pre-burst time and the whole dynamics of phage spread is influenced by the history of the infected colony from which the founder cell derives. Aged, non-productively infected cells could be increasingly committed to support lytic cycles that would take place when nutrients become again available by re-culturing on fresh medium, after pre-burst times that become progressively shorter.

Conclusions of the Characterization of Peptide-Displaying P22 as Immunobiosensor

8. In the case of the β-galactosidase molecular sensors, distinct mechanisms of antibody-mediated enzymatic reactivation has been showed:
   i. The β-galactosidase M278VP1-antibody complex showed a decreased k_m with an unmodified k_{cat} (class I). Reactivation of M278VP1 was observed with antibodies 3E5, but didn't respond to the 3E5 Fab fragment, indicating that bivalent interaction might be required for activation of the enzyme. The inserted peptide could cause steric impediments to the access of substrate and the bivalent interaction of the antibody binding could reduce these steric impediments and allow higher substrate diffusion.
   ii. The β-galactosidase JX795A antibody complex showed an unmodified k_m with an increased k_{cat} (class II). Reactivation of JX795A observed with immune guinea pig sera, could be due to an increase in the actual number of active molecules promoted by the antibody, which might result from tetramer stabilisation and the consequent displacement of the monomer-tetramer equilibrium.

9. On the other hand, it has been demonstrated that chimeric P22 particles assembled *in vitro* of tailless P22 heads with recombinant TSPA (TSP in which a FMDV antigenic peptide has been joined to its carboxy terminus) are able to detect the presence of Fab fragments anti-Site A of 3E5. Binding of an anti-peptide specific Fab antibody fragment increases the titre of infectious chimeric bacteriophage particles by more than 500 % and the increase is dependent on Fab concentration.

10. The reactivation by 3E5 Fab increases concomitantly with the molar amount of TSP, reaching a level of 900 % when an average of 105 copies of TSP trimer are added per head.

11. It has been shown a decrease of infectivity in TSPA particles that is also depending on tail protein concentration. At conditions representing a TSPA molar excess respective to phage heads, the remaining infectious particles are less than 5 %.

12. The binding of the antibody fragment would restore only the conformational defects promoted by the inserted peptide, recovering viral infectivity up to the titre exhibited by P22TSP phages.

13. Modeling approaches have demonstrated that there aren't sterical restrictions to the binding of three antibody fragments to one TSPA trimer.

14. In conclusion, peptide-displaying bacteriophage P22 is proposed as a new immunobiosensor to detect molecular interactions, through a viral enzyme critical for bacterial infection.

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