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Mycobacteriophages as versatile tools for genetic manipulation of mycobacteria and development of simple methods for diagnosis of mycobacterial diseases

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ABSTRACT

Tuberculosis, caused by *Mycobacterium tuberculosis*, is responsible for over two million deaths per year worldwide. Due to its long doubling time (18 h), the microbiological detection of *M. tuberculosis* by conventional methods takes up to one month, unless the number of bacilli in the biological sample is high enough. Thus, drug resistance assessment requires at least one month for obtaining the primary culture and another month to determine its susceptibility to anti-mycobacterial drugs. Moreover, for a long time, the lack of genetic tools for mycobacteria has been a barrier for undertaking studies aimed at understanding the mechanisms of drug resistance and drug target identification, being all these topics of utmost importance considering the increase in the number of drug-resistant clones and the few therapeutic options available. Mycobacteriophages are promising as a novel source of genetic elements for mycobacteria manipulation, as well as for the development of versatile, simple, fast and cheap methods for drug resistance assessment of *M. tuberculosis* clinical isolates. We herein describe the background related to the use of mycobacteriophages, with emphasis placed on their utilization for drug resistance analysis in our country.

Key words: mycobacterial genetics, mycobacteriophages, diagnostic tools, tuberculosis

RESUMEN

Micobacteriófagos como herramientas versátiles para la manipulación genética y el desarrollo de métodos simples para el diagnóstico de enfermedades micobacterianas. La tuberculosis, enfermedad causada por el bacilo *Mycobacterium tuberculosis*, es responsable de más de dos millones de muertes anuales en el mundo. Debido a su largo tiempo de duplicación (18 h), la detección bacteriológica de *M. tuberculosis* por métodos convencionales necesita de un mes o aun más, a menos que el número de bacilos en la muestra clínica sea suficientemente alto. Por consiguiente, se necesita un mínimo de dos meses para determinar la resistencia de este microorganismo a las drogas antituberculosas: uno para obtener el cultivo primario y otro para ensayar la sensibilidad frente a aquellas. La falta de herramientas para la manipulación genética de micobacterias ha dificultado la identificación de los blancos de acción de las drogas y el estudio de los mecanismos de resistencia a éstas, tópicos de la mayor relevancia dado el aumento mundial del número de aislamientos clínicos multirresistentes y las pocas opciones terapéuticas disponibles. Los micobacteriófagos son considerados nuevas herramientas para la manipulación de las micobacterias, así como para el desarrollo de métodos simples, rápidos y económicos para determinar la sensibilidad a drogas de los aislamientos clínicos de *M. tuberculosis*. En esta revisión se describen los antecedentes del uso de micobacteriófagos con énfasis en su utilización para el análisis de resistencia a drogas antituberculosas en nuestro país.

Palabras clave: genética de micobacterias, micobacteriófagos, herramientas diagnósticas, tuberculosis

INTRODUCTION

Tuberculosis (TB) is a major cause of illness and death worldwide, especially in Asia and Africa. Far from being eradicated, tuberculosis has re-surfaced, riding on the back of famine and poverty and the HIV pandemic, leading the World Health Organization (WHO) to declare tuberculosis a global emergency in the mid ´90s. A number of programs have been designed with the aim of stopping and reversing the incidence of tuberculosis by the year 2015 (40, 41, 65, 87). As detailed in several WHO publications, some of the goals have been achieved while others are evading control in spite of the massive budget available for TB control (US$ 3.3 billion across 90 countries accounting for 91% of global cases). Globally, an estimated 9.2 million new cases and 1.7 million deaths from TB occurred in 2006, of which 0.7 million cases and 0.2 million deaths corresponded to HIV-positive people, with an estimated 0.5 million cases of multi-drug- resistant TB (MDR-TB) (43, 87).

Among the number of reasons that sustain the spread of tuberculosis in the world, we must mention the consequences of global economy-driven immigration, failing national tuberculosis programs and lack of knowledge of the tubercle bacillus as a microbe, since until recently, information on its genetics and physiology has been scarce.

Some of the WHO recommendations aim at global TB control, patient management, laboratory service and research and development (32, 35, 68). Regarding this last point, the utilization of mycobacteriophages has been a driving force to pierce the shield of mycobacteria by becoming the basis for the development of tools for the molecular analysis of these bacilli. This review intends to illustrate some of the valuable results of the research on mycobacteriophages.

BACTERIOPHAGES: THE CRUSHING WEIGHT OF NUMBERS

Bacteriophages (phages) are prokaryotic infecting viruses and the most abundant organisms in biosphere, according to Bergh *et al.* roughly 10^{30} phages (9). They play important roles in keeping the balance of microbial communities, not only because during infection, bacteria may be killed by the ensuing phage replication but also because phages are a reservoir of genetic information and, therefore, are providers of genetic variability to the infected host. Since no two identical phages have been identified so far, it may be concluded that genetic variability is extremely large and, as in the rest of the biological universe, phages may undergo expansion and contraction of their numbers and families depending on the variation in the numbers of their preferred microbial hosts. Due to their small genomes [generally less than 150 kilobase pairs (kbp), with an average of 40-70 kbp] and the simplicity of their amplification, phages, in general, have been marvellous tools for the analysis of biological processes such as genetic recombination and transfer of genetic information, and because of this ability, for the artificial genetic manipulation of bacterial species in order to study various aspects of their physiology (9, 16, 17, 20, 50, 51).

After infection, phages may follow three different paths: one is the "lytic" cycle: their replication lead to an increase in the mass of phage proteins and nucleic acids which will finally be assembled by different mechanisms prior to the lysis of the bacteria and the release of the phage progeny; a second route and quite an important one for molecular biologists and geneticists the "lysogenic" cycle, is the reversible integration of the phage DNA into the bacterial genome. The third path is the "pseudo lysogeny" a seldom detected phage strategy, which we will not consider further.

For the purpose of integrating into the bacterial chromosome, phages contain DNA sequences highly homologous to a specific chromosomal sequence present in the bacteria which the phage infects, which are known as "*att*achment sites", represented as *att*B and *att*P for bacteria and phages, respectively. These sequences vary between different phages and bacteria, therefore leading to a universe of possible integration events, provided that two *att* sequences (B and P) are similar enough. Thus via site-specific recombination, the phage genome is integrated within the bacterial chromosome at a precise location. The phages that follow that path are called temperate phages, the process is known as lysogeny and the integrated phage is called a "prophage". In this way, the phage replicates synchronously with the bacterial genome, being able to excise out of the chromosome, provided that specific signals (such as DNA damage) are sensed. Once the prophage is induced, a lytic cycle will follow, producing lysis of the bacterial cell and release of the infective particles.

Regardless of the temperate or lytic nature of the phage, once the structural constituents (capsids and tails and replicated DNA) are ready in the bacterial cytoplasm, there is a coordinated assembly of capsid and tails along with DNA packaging. At this point, it will be important, for the sake of future references in this review, to define two strategies for the packaging. The first one consists in the "headful" mechanism in which the assembled virion "loads" a determined amount of DNA, which could be self or bacterial DNA that has been chopped down by endonucleases. In this way, a percentage of the released virions would carry bacterial DNA and thus, the phage would act as a generalized transducing phage, since it could deliver different regions of bacterial DNA to a susceptible bacterial host with enough chromosomal homology to allow for genetic recombination. This useful tool has allowed the researcher to "move around" different alleles of a gene among strains (62, 99, 101), thus enabling the construction of strains bearing a combination of precisely defined mutant alleles. In consequence, through a new round of infection of a permissive host, the infective phage particles may lead to a swapping of gene alleles in the bacterial chromosome.

The second strategy occurs in phages that use a replication mechanism that produces subsequent multiple copies of their DNA in a single molecule ("concatemers"). The packaging mechanism is based on the recognition of a defined sequence in the amplified phage DNA which is present in each phage genome monomer. This sequence works as a "stop" signal and is associated with a nuclease that recognizes and cuts the DNA at the signal while packaging. That translates into the packaging of one genome of "self" DNA that fits into the head of the virion.

Perhaps, the most important of these two strategies for the geneticists and molecular biologists is the second one, since, due to the specificity of the nucleic acid packaging into the assembled phage capsid, a phage progeny may contain only phage DNA inside the virions, un-

less it is artificially manipulated or errors in the excision of the prophage take place.

Phage lambda, one of the most studied phages, has yielded large amount of information on the molecular biology of the process of virion assembly, as well as helped define the host´s cellular components that are needed for a successful production of phage progeny. At the same time, the knowledge gained from the analysis of its genome organization and regulation of gene expression has allowed the use of lambda for three very advantageous applications:

1- the construction of genomic libraries into lambda derived vectors lacking non-essential genome sequences, thus capable of accommodating a few kbp of foreign DNA (22, 36, 111), and upon infection, the integration of the lambda derivative into a precise chromosomal spot of otherwise non-integrative cloning vectors, leading to the expression of cloned genes without gene dosage effect;

2- the construction of plasmid vectors containing the sequence coding for the lambda DNA packaging system (known as a “cos” site, and thus, by extension, the “cos” containing plasmid is dubbed a “cosmid”) for cloning and packaging of large fragments of any chromosomal DNA (52, 53, 55, 56), and

3- the construction of an *in vitro* packaging kit with purified heads and tails for packaging “your favourite source of DNA” in a compatible form (such as cosmids containing an amount of DNA of any source with a size within the packaging range of the phage) (54, 57). Thus, genomic libraries of any organism could easily be made and amplified by infection of a suitable bacterial host for further studies.

As will be described below, these basic principles have been wisely extrapolated from the *E. coli* and lambda phage to mycobacteria by the work from G. Hatfull and W.R. Jacobs.

USING MYCOBACTERIOPHAGES TO BREAK OPEN THE MYCOBACTERIAL PANDORA BOX

Although phages able to infect mycobacteria were reported several years ago, they were mainly used for typification purposes (13, 23, 61) –except for the very early work by Y. Mizuguchi on lysogeny by mycobacteriophages, mostly only available in Japanese language– until Dr. Bill Jacobs and Dr. Graham Hatfull joined forces to tackle the genetic analysis of *M. tuberculosis*. By the early ´90s, although the chemical composition of the lipid-rich cell envelope of this pathogen had been analyzed in detail, and there were several studies on the immunological and cell biology aspects of the infection, up to that moment there had been no means to genetically study the tubercle bacilli or any other mycobacteria. The lack of natural or chemically induced competence for transformation was one of the reasons for this road block. Jacobs and Hatfull assumed that –as has happened in other bacterial systems– phages may provide regulatory signals and genetic elements that could be used for vector design. Thus, they started to look for novel mycobacteriophages in the environment and, while embarking in a very fruitful “phage-hunting” program (81), they constructed cloning vectors containing promoters of mycobacteriophages L5 and D29 (80, 98). Those cloning vectors, which were rapidly adopted by the scientific community, constituted the first generation of vectors for genetic manipulation of mycobacteria, although suitable conditions for transformation through electroporation had to be developed for their widespread utilization (45, 97).

Temperature-sensitive derivatives of phage TM4, deficients in replication at high temperature, were also obtained, allowing the regulated delivery of transposons –carrying genes encoding for resistance to antibiotics– into the mycobacterial chromosome (12). In that way, random insertions which may cause auxotrophies could easily be obtained. Since the genetic sequence of the transposon is known, junction DNA sequences could be obtained with little difficulty leading to the identification of chromosomal position of the insertion site. Therefore, insertional mutants of *M. tuberculosis* H37Rv (a virulent lab strain) could be used for infection of susceptible animal models such as mice or even in vitro infection of macrophages, which is the usual niche for this pathogen. Thus, the place –and therefore the gene– in the genome where the transposon was inserted could be identified, allowing for the first studies focused on the impact of the loss of specific genes in the virulence of *M. tuberculosis* (58, 59, 92-94).

Through a highly defined PCR-based cloning system in a cosmid, Hatfull and Jacobs were also able to make precise deletions in any gene of the mycobacterial genome, provided that the sequence of adjacent DNA was known (11). That was the first time in which “custom-made” mutants were obtained in mycobacteria by using mycobacteriophages (although it was also accomplished at the same time by the utilization of suicide plasmids by Gicquel´s group at the Institut Pasteur), thus opening the door for an exhaustive functional and structural protein analysis. Moreover, as mentioned above, mutants carrying specific gene deletions could be tested for their virulence attenuation, helping to pin down the genes involved in pathogenesis.

Thus, phages were not only a successful and handy tool-box for development of cloning vectors but also a scaffolding used for strain construction by means of transduction of chromosomal fragments. Unfortunately, that could only be achieved in the non-pathogenic saprophytic *M. smegmatis*, since so far no generalized transducing phage has been described for *M. tuberculosis*. From that point on, the knowledge gathered by the application of these novel phage-based technologies has been breathtaking, leading –among several other examples– to the understanding of the differences during the onset of the

infection between the pathogenic *M. tuberculosis* and the vaccine strain *M. bovis* var. BCG, the identification of most of the targets for currently used anti-tubercular drugs and epidemiological studies on the distribution of strains with different level of virulence (14, 39, 59, 109, 114).

As mentioned before, the quest for novel tools for genetic analysis led these researchers to a phage-hunting program that was used for undergraduate student mentorship in biological sciences (47). During that exciting period, 50 new phages were isolated, sequenced and characterized, giving copious amounts of information on gene organization and horizontal gene transfer in mycobacteria (37, 38, 46, 66, 67, 74, 82, 83). The genetic analysis of those newly isolated phages showed high diversity, but also a remarkable degree of mosaicism, product of illegitimate recombination (81). Hatfull *et al.* (46) recently reported a summary of the information obtained in the analysis of 37 of those 50 new mycobacteriophages, pointing out that all of them were double-stranded DNA phages, most of them belonged to the group of *Sipho-virida*e phages with long flexible non-contractile tails, while a few belonged to the group of the *Myoviridae* phages that contain contractile tails. Most of the mycobacteriophages showed isometric heads at the electron microscope, although some of them (e.g. Che9c, Corndog) exhibited large elongated heads. While lytic genetic cassettes (encoding for enzymes involved in "poking" holes in the bacterial cell membrane to help virion release) have been detected in the majority of the sequenced phages, integration components (*att*P sites and recombinases) are roughly present in half of them (46). A very interesting outcome of those studies is the detection of proteins involved in recombination, some of which are homologous to the well-known RecA bacterial protein. In a strikingly important and elegant approach, the Hatfull´s group has recently developed a method based on recombinogenic enzymes from phage Che9, which might soon replace the specialized transduction method (104-107). The "recombineering" method avoids the costly *in vitro* cosmid packaging, thus it is much cheaper and will soon become the preferred method for creating specific deletions in the mycobacterial chromosome.

Although more studies are needed, it is clear that phages are a treasure chest, which will be providing a large number of genetic elements for mycobacteria manipulation for a long time.

MYCOBACTERIOPHAGES AS DIAGNOSTIC TOOLS

Pioneering work by Hugo David in the early´80s showed that phage D29 was able to infect *M. tuberculosis* as well as *M. smegmatis*, but not *M. avium* (26). During the characterization of its infective cycle, Dr. David wisely foresaw the possible application of that phage for the assessment of drug resistance in clinical isolates of *M. tuberculosis* (25). The principle was that aliquots of *M. tuberculosis* cultures from clinical isolates treated with anti-tubercular drugs blocking macromolecular synthesis [such as streptomycin (STR) and rifampicin (RIF)], would not allow for the replication of added D29 phages, unless the clinical isolate was resistant to that drug. Thus, by performing a simple phage amplification assay on bacilli cells treated with drugs or left untreated, one could distinguish between drug-susceptible and drug-resistant *M. tuberculosis* isolates (see Figure 1 for a scheme). Therefore, phage amplification would quickly be visualized by a second round of infection of the fast-growing *M. smegmatis*, thus reducing the time-around to inform drug susceptibility from 45-60 days, by conventional bacteriological means, to four days. For this approach to be consistent, the fraction of added phages not adsorbing to the tubercle bacilli had to be eliminated to avoid false positives, since these phages would also give lysis on *M. smegmatis*. The problem was solved by Mc Nerney *et al.* (73), who found a chemical compound (ferrous ammonium sulphate) that would eliminate unabsorbed virions, so that only phages finding viable *M. tuberculosis* cells would be able to yield phage progeny because their internalization would protect them from the chemical "virucide". Further studies by McNerney *et al.* (70, 71), Wilson *et al.* (112) and Elthringham *et al.* (33) laid the foundation for the use of D29 for drug susceptibility determination in *M. tuberculosis* isolates. Thus, a novel method based on mycobacteriophages was available for the test.

On the other side, Jacobs and collaborators continued their development of phage derivatives aiming at *M. tuberculosis* detection as well as drug susceptibility testing, but instead of using the lytic D29 phage, they preferred to use a genetically modified derivative of the tem-

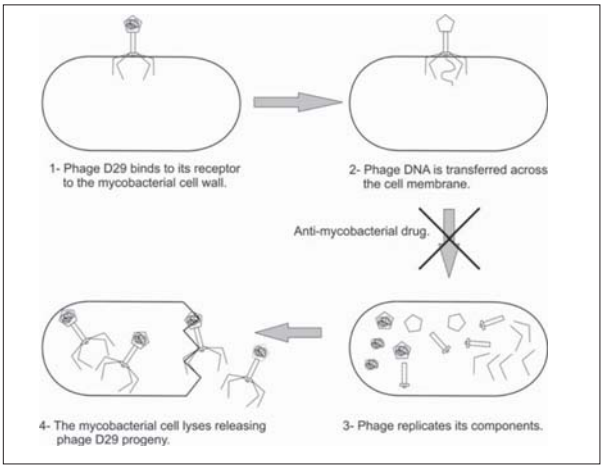

Figure 1. Basis of the D29-based assay. The scheme shows adsorption of the phage particle, injection of phage DNA into the mycobacterial cell and phage replication. Treatment of a drug-susceptible *M. tuberculosis* clinical isolate with appropriate drugs will kill the bacilli, thus inhibiting the phage replication. On the contrary, a drug- resistant *M. tuberculosis* clinical isolate would survive allowing the phage replication.

perate mycobacteriophage TM4 that had previously been isolated from a *M. avium* strain. In a very elegant approach, these researchers cloned the firefly luciferase (*lux*) gene in a dispensable region of the TM4 and L5 (60, 80, 88, 89, 91). Luciferase is an enzyme responsible for converting the enzyme substrate luciferin-into a derivative with subsequent light emission. This reaction, when performed *in vitro*, requires the addition of ATP, but this technology would only work in whole cells (since ATP cannot enter the cell) if the mycobacterial cell is viable and therefore synthesizes its own ATP. In consequence, "light emission" was equivalent to viable *M. tuberculosis* cells and the strategy of treatment with anti-mycobacterial drugs was still appropriate. Since the protocol called for determination of the emitted light, and therefore, the placement of a luminometer inside a biosafety cabinet would be required, the Jacobs´ group went one step further by developing the "Bronx Box", a closed system based on 96 well microtiter plates on top of which a Polaroid film system was assembled (6, 7, 10). Each well would contain aliquots of *M. tuberculosis* cultures with the addition of different drugs and a Polaroid film would be exposed during the incubation of the device (manipulation of the Bronx Box had to be performed in a biosafety cabinet). In that way, light emission could be detected from the wells where productive infection of viable bacilli took place.

In spite of the very solid conceptual development, and the possibility of signal amplification through electronic devices and image manipulation, the TM4- based method was only tested by the group that developed it, although with good results for drug resistance determination (turnaround time 2 days, sensitivity for detection of resistance to RIF and INH 100%) (6-8, 10, 48).

Some of the reasons for the preference of the D29-based method as opposite to the TM4-based one are the following: a- D29 kills bacilli, rendering the sample safe from a biosafety point of view; b- no addition of a substrate is needed since there is no reporter gene and; c- there is no need for costly equipment. Perhaps, these reasons explain in part the preference of D29 in several laboratories and multi-centric tests, as will be discussed in the next section.

PCR-BASED METHODS FOR DETECTION OF *M. TUBERCULOSIS* AND DRUG RESISTANCE ANALYSIS

Tuberculosis is perhaps one of the very few situations at a microbiology lab when the utilization of PCR-based methods does not solve the question of the presence of a viable microorganism causing disease, as well as its drug susceptibility. Since the "culprit" of the disease has to be isolated from microbiological media and, at least a month is required to produce visible colonies on solid bacteriological media (that are needed to subsequently perform drug susceptibility testing using the gold standard method), there is an urge for a quicker way to detect viable bacilli to confirm a positive detection of mycobacterial DNA by PCR. In addition, specificity issues were a concern at the early stages of the use of molecular biology methods, because PCR is capable of detecting few copies of a target nucleic acid, but extreme care has to be taken to avoid false positives due to the presence of DNA from environmental non-pathogenic mycobacteria to which we are constantly exposed. Even when the information gathered in the last ten years of research allowed for the identification of sequences only present in *M. tuberculosis* (14), by using a PCR-based method, we may only say that the cause of sickness is very likely to be that pathogen, especially if clinical symptoms are compatible with tuberculosis. Thus, the utilization of a PCR-based method for direct detection of *M. tuberculosis* in sputum is an unsettled controversy due to the number of different in-house protocols used and the variable results published. In spite of that the method may safely be used to confirm that a primary isolate is of *M. tuberculosis* and not of a non-tuberculous mycobacteria (NTM) by using well-established gene markers such as IS6110 (15, 30, 49, 69, 84, 85).

In spite of that, according to a recent meta-analysis, in-house PCR-based methods has limited value for the diagnosis of pulmonary tuberculosis (42), although they keep their potential for extra-pulmonary cases where bacillary counts are very low but the sample is less toxic to PCR protocols. Cerebral spinal or pleural fluids are two of such cases, as both pleural tuberculosis and tuberculous meningitis are extremely dangerous situations where a rapid diagnostic improves the chances of saving the patient's life (15, 86).

Although it is beyond the scope of this review, we should briefly address the issue of drug resistance mechanisms in mycobacteria. This field has been one of the most benefited by the number of advances in genetic analysis. So far, the mechanisms of action and of resistance to common anti-bacterial drugs, such as rifampicin (RIF) and streptomycin (STR), that are also used for the treatment of tuberculosis have also been determined in mycobacteria. Similar studies were conducted leading to an understanding of the novel mechanisms of action of drugs specifically active against mycobacteria. Surprisingly, most of the currently used anti-tubercular drugs are in reality pro-drugs that need to be activated to produce the inhibitory effect (31, 110). That is the case for isoniazid (INH) and the related molecule ethionamide (ETH), as well as for thiacetazone (TAC), isoxyl (ISO) and pyrazinamide (PZA). Therefore, we may think of at least three factors leading to resistance to those drugs: loss of the activating protein, mutations in the target for the active molecule and efflux mechanisms that pump out pro-drugs (29, 31, 34, 75, 108, 110, 114). In spite of the great deal of research, there are several reports of clinical strains with resistance phenotypes, for which no mutations were

detected in the genes, known to be implicated in the action of the drug. As an example, there are reports of *M. tuberculosis* INH-resistant strains for which no mutation has been found in any one of the four known genes involved in the INH-resistance phenotype. Thus, it would be a daunting task to use a PCR protocol to determine whether a clinical strain is susceptible or not to a drug because of the considerations mentioned above. As a result, when performing the detection of mutant alleles linked to drug resistance, a negative PCR must not be interpreted as lacking a resistant phenotype, so therefore, the fundamental question of drug resistance evaluation still requires a better method. If we think of performing the analysis directly on the sample (which, in most cases is sputum, since pulmonary tuberculosis is the most frequent form observed) we have to consider that, besides the mentioned facts, PCR is also known for its vulnerability to inhibitors of different nature present in the clinical sample. Since sputum is not sterile and contains a large amount of cell debris, proteases, and various other macromolecules, the inhibition of the PCR reaction is a very possible outcome (21, 102). Thus, in spite of its versatility and sensitivity, PCR may not be a good method for the determination of drug resistance in *M. tuberculosis*, taking into consideration the problems inherent to the nature of the biological sample and the large number of genes involved in resistance to some of the currently used anti-tubercular drugs. However, as novel methods are being developed and tested, a way to overcome these problems may be feasible.

TESTING OF D29-BASED METHODS IN LATIN AMERICAN CLINICAL SETTINGS

It did not take too long from the development of the D29-based method to implementing its testing in a number of mycobacteriology reference laboratories in Latin America, thanks to the help provided by Dr. Juan Carlos Palomino and Dr. Ruth McNerney (at the Institute of Tropical Medicine, Antwerp, Belgium and the London School of Hygiene & Tropical Medicine, Keppel Street, London, U.K., respectively), the driving force for that project. Both researchers were confident of the performance of this very fast and cheap method in our local medium- or low-resource clinical settings. It was already a well-known fact that a large fraction of RIF- resistant clinical isolates would also be resistant to INH as well (43). These two anti-tubercular drugs are the most important ones in the therapeutic scheme usually employed in our countries, being maintained during the two phases of the treatment. Therefore, resistance to one or both drugs would seriously compromise the treatment outcome and the patient recovery. The fast detection of clinical isolates bearing mutations causing RIF and INH resistance would, then, be of utmost importance.

With these needs in mind, several laboratories tested the D29-base method to determine the status of RIF resistance in *M. tuberculosis* clinical isolates. The published results compared the performance of the D29 method (also called PhaB, Phage Amplified Biologically Assay) against the gold standard method (bacteriological culture in Lowenstein Jensen medium -also known as Proportion Method, PM), as well as against two other rapid methods, the REMA [Resazurin Micromethod Assay, (78)] and the BacTec or MGIT systems (90). The results reported in those publications showed that PhaB and REMA could be used to shorten the turn-around time, with good sensitivity and specificity but being much cheaper than costly methods such as the BacTec and MGIT (1, 5, 19, 24, 72, 113).

The PhaB method has been used in several countries (5, 18, 19, 64) and has also been the subject of two meta-analysis highlighting the interest of the researchers on this method (63, 76, 77). In our country, the team led by Dr. L. Barrera (INEI-ANLIS Instituto Nacional de Microbiología “Dr. Carlos G. Malbrán”) pioneered the use of D29 for the determination of RIF resistance with great success (95). Unfortunately, most of the published tests were performed with the focus on the analysis of RIF resistance and, only in very few cases on INH or STR resistance. Thus, our laboratory assayed PhaB to analyze RIF, INH and STR resistance (28); and more recently fluoroquinolone resistance (FQs). Our results agreed with previous publications highlighting the relative simplicity, low cost and biological safety of this method. While drug susceptibility testing by the gold standard method (PM) starting from primary cultures, takes 28-42 days in Lowenstein-Jensen culture medium and 21days in 7H11 medium (results may be obtained within 7-12 days when vital dyes are used), PhaB results can be obtained at 48, 72, and 96 h, respectively, when susceptibility to RIF, STR and INH is assayed. The sensitivity and specificity of the method (with a 95% confidence interval) were as follows: PhaB-RIF 97.7% (86.2-99.9), 100% (84-100); PhaB-INH 93.61% (81.43-98.33), 100% (81.50-100); and PhaB-STR 96% (85.14-99.30), 100% (97.07-100). Thus, these results are quite encouraging and show that PhaB may be used for drug susceptibility analysis in clinical isolates of *M. tuberculosis*. Some of our results are shown in Figure 2. Unfortunately, our results suggest that PhaB may not be a good choice to assess FQs resistance, since the time required to obtain a reliable result without major modifications of the protocol was comparable to that obtained when using REMA (seven days). Therefore, for the purpose mentioned and under its current protocol, PhaB offers no advantage when compared to REMA (Stella E.J., personal communication).

Although PhaB is not a validated method and therefore, cannot be informed to the treating Infectious Disease fellow, it would be reasonable to expect a worldwide multicentric study for its validation on the basis of

the extraordinary time reduction, from the 30-45 days required for conventional microbiological technique to the two to four days mentioned above.

PHAGE-BASED METHODS: WEAKNESSES TO ADDRESS

In spite of the obvious advance in the determination of drug susceptibility of clinical strains of *M. tuberculosis* produced by the development of methods based on the utilization of simple reagents such as mycobacteriophages, there is an unfulfilled promise in those methods, which is their ability to detect viable tubercle bacilli directly in clinical samples. A commercial kit based on D29 has been tested but the results were not as satisfactory as should be expected from a commercial source, including false positives and not interpretable results (2-4, 96, 100). Fortunately, the study of sputum smears by optical microscopy is still the best choice for detection of tubercle bacilli directly in the sample. A simple and cheap stain (acid-fast or Ziehl Neelsen stain) that takes advantage of the uniqueness of the lipid-rich composition of mycobacteria, will clearly reveal its presence in sputum. This method is limited by the number of bacilli present in the sample, having a sensitivity of 10^4 bacilli/ml of sputum. All samples with no microscopically detectable bacilli may require bacteriological confirmation, especially if the clinical symptoms are suggestive of pulmonary tuberculosis. Those samples that have detectable numbers of *M. tuberculosis* will require the evaluation of the drug resistance status, a practice indicated when that patient has risk factors (such as previous treatment, contact with confirmed tuberculosis cases, or time in institutions such as prisons or mental health facilities). At this point, we face again the same problem: the need for a rapid method that will not require the 30 days needed to obtain the primary culture and the 30 - 45 days required to perform the anti-tubercular drug testing. As we have described, PhaB can be effective for the determination of drug susceptibility in primary cultures, but why is that it does not work in direct samples? A very likely explanation resides in the chemical and biological complexity of sputum: in that biological fluid, a matrix of high molecular weight glycoproteins traps the tubercle bacilli in the sputum (27, 44, 103); along with the bacilli, other bacteria from the upper respiratory tract and mouth, as well as intact and lysed cells from the host respiratory tract are present. To add complexity to this scenario, lysed cells provide large amount of proteins, carbohydrates and enzymes that may be harmful to the phage, such as proteases, and other macromolecules as glycoproteins, that may interfere with phage adsorption to mycobacteria. Thus, it is extremely unlikely that a phage particle would be able to find its prey within this matrix while avoiding both the chemical and enzymatic “insults” from the materials in the sample.

A PhaB based protocol developed by Park *et al.* (79) was tested in mock samples (sputum samples from healthy volunteer donors to which known quantities of *M. bovis* var BCG had been added). According to their published results, it was capable of detecting 10^3-10^4 CFU/ml of sputum, which is about the same sensitivity demonstrated by optical microscopy. Unfortunately, our lab could not reproduce those good results in several attempts using the Park´s protocol. Moreover, to the best of our knowledge, the protocol had not been tested in any other laboratory. Notwithstanding those facts, phage-based methods would be usable in direct samples if the rheological features of the sample could be adequately modified, a task that our group is currently engaged in.

MYCOBACTERIOPHAGES “MADE IN ARGENTINA”: ADDING ANOTHER BRICK TO THE WALL

Due to the simple techniques required for phage isolation, that make phage biology an attractive subject for undergraduate work, we have started our own “phage–hunting” program, with the goal of isolating phages, sequencing their genomes and utilizing them as tools to study mycobacterial cell envelope organization. By using the saprophytic fast growing *M. smegmatis* as a host, we searched for phages in soil samples from different geographic places in Argentina. As a result, we isolated 17 mycobacteriophages, five of which were characterized as temperate ones. We performed Transmission Electronic Microscopy (TEM), which allowed us to analyze the structural features of the virions; as expected, a large variety of head morphologies and tail lengths was found. As an example, TEM of two phages, D29 (used in the PhaB assay) and F (isolated in our laboratory) are shown in Figure 3. The genome sequencing of the newly isolated temperate mycobacteriophages will allow us to develop our own series of integrative cloning vectors which will increase the already available variety of vectors of this type, since chances are that their chromosomal integration sites will be different from those already reported.

One of the most rewarding aspects of our current research in the field of mycobacteriophages has been the isolation of phage resistant *M. smegmatis* mutants with alterations in the cell envelope composition, as evidenced by changes in colony morphology, sliding motility and biofilm formation. To this end, a combination of simple techniques and culture media were used; i.e. the addition of the Congo Red dye to rich medium plates allowed us to detect morphology alterations as well as changes in the intensity of the color of the colony, due to the increase in the amount of bound dye which preferentially binds glycolipids (results from one of these phages are shown in Figure 4). Thus, by using simple techniques, we are able to start studying complex biological features of mycobacteria, such as cell envelope composition with the ulti-

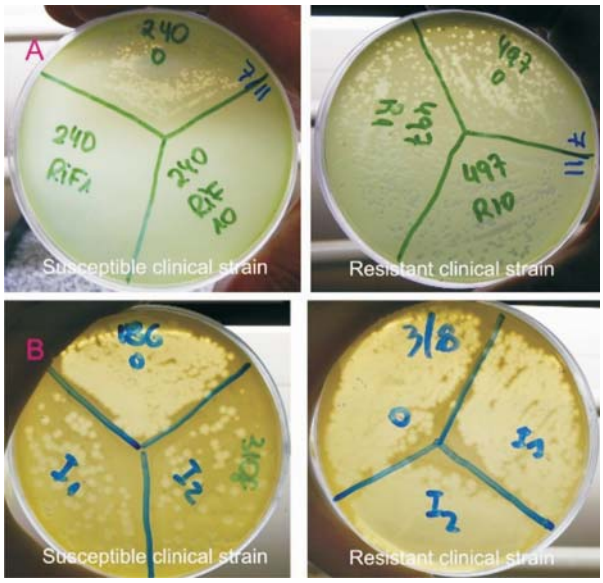

Figure 2. After the phage addition and an adequate incubation time, an aliquot of the mixture of mycobacteria and phage is plated on top of indicator plates containing a lawn of *M. smegmatis* cells. Lysis of indicator cells is easily scored after 24 h of incubation. Panel A: left, RIF-susceptible strain; right, RIF-resistant strain. Panel B: left, INH-susceptible strain; right, INH-resistant strain. Drug concentrations marked on the plate surfaces are as follows: RIF, 0, 1 and 10 μg/ml; INH, 0, 1 and 2 μg/ ml.

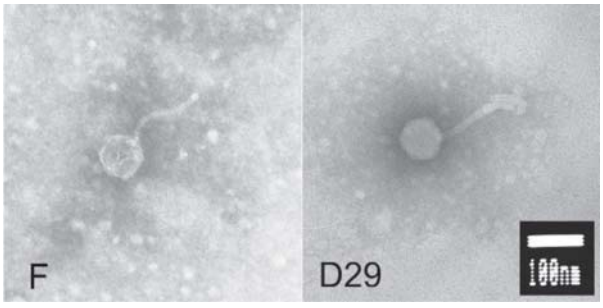

Figure 3. Transmission Electron Microscopy. Samples were analyzed in a Jeol 1200 EX II Electron Microscope at 85KiloVolts, with a magnification of 50,000X.

mate goal of identifying mycobacteriophage receptor(s), a research field for which information is extremely scarce.

In conclusion, by "bursting into" the molecular biology field of mycobacteria, mycobacteriophages have reached the status of powerful allies to both researchers and clinical microbiologists. There is little doubt that their ensuing application will allow the genetic analysis of mycobacteria towards the ultimate goal of unveiling the intimacies of the host-pathogen interaction, which will in turn help to design novel anti-mycobacterial drugs and better vaccines.

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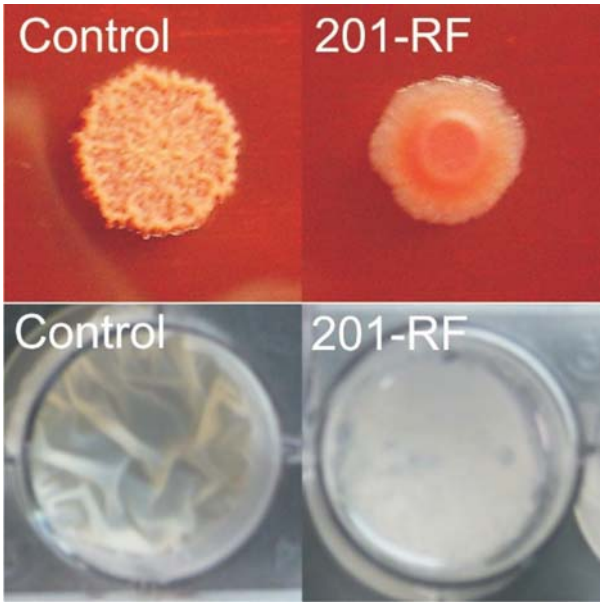

Figure 4. Impact of a phage resistance phenotype on *M. smegmatis* cell envelope. Upper panel, Congo Red medium; lower panel, biofilm formation. Left panels, *M. smegmatis* strain mc^{2}155 (parental strain); right panels, mutant resistant to phage F.

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