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A Phage-Guided Route to Discovery of Bioactive Rare Actinomycetes

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1. Introduction

The discovery, development and exploitation of antibiotics was one of the most significant advances in medicine in 20th century and in a golden era lasting from 1940s to late 1960s, antibiotic research provided mankind with a wide range of structurally diverse and effective agents to treat microbial infections (Table 1) (McDevitt and Rosenberg, 2001; Hopwood, 2007). However, antibiotic resistance has developed steadily as new agents have been introduced and there has been a dramatic increase in the occurrence of resistant organisms in both community and hospital settings for the past 10-15 years. In particular, pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* and *Enterococcus faecalis* capable of resulting in severe and fatal infections have become increasingly resistant to multiple antibiotics. In hospital and community environments, Methicillin-resistant *S. aureus* (MRSA) and Vancomycin resistant enterococci (VRE) have become persistent pathogens. Other multiple drug resistant organisms currently include *Mycobacterium tuberculosis* and *Pseudomonas,* and related species in the hospital environment. Last line of antibiotics such as vancomycin might also become ineffective against super-bugs such as vancomycin-intermediate-resistant *S. aureus* isolates. New classes of antibiotics with a new mode of action (e.g. Linezolid™) are necessary to combat existing and emerging infectious diseases deriving from multiple drug resistant agents (McDevitt and Rosenberg, 2001; Hopwood, 2007).

An extreme example for yet to be faced outbreaks has been the recent occurrence of multi-drug resistant enterohaemorrhagic *E. coli* in Germany claiming the lives of many (Chattaway *et al*., 2011). Interestingly, this strain acquired virulence genes from another group of diarrhoeagenic *E. coli,* the enteroaggregative *E. coli* (EAEC), which is the most common bacterial cause of diarrhoea. This event once more stressed the importance of powerful diagnostic systems to detect all diarrheagenic *E. coli* as part of routine surveillance systems, which would thus contribute to the mapping of the global distribution of EAEC (Chattaway *et al*., 2011; Mellmann *et al*., 2011).

For more than a century, infectious diseases have been controlled by vaccination and the administration of antibiotics (Muzzi *et al.,* 2007). In spite of the technical progress of the past century, innovation in both fields came exclusively from traditional approaches, and antibiotics have been identified by screening natural compounds for their ability to kill

DISCOVERY YEARS	NAME OF ANTIBIOTICS
1940-1950	Streptomycin Streptothricin Actinomycin
1950-1960	Neomycin Chlorotetracycline Candicidin Chloramphenicol Spiramycin Tetracycline Erythromycin Oxytetracyline Nystatin Kanamycin
1960-1970	Mitomycin Novobiocin Amphotericin Vancomycin Virginiamycin Gentamicin Tylosin Pristinamycin Polyoxin Rifamycin Bleomycin
1970-1980	Monensin Adriamycin Avoparcin Kasugamycin Fosfomycin Bialaphos Lincomycin Teicoplanin
1980-1990	Thienamycin Rapamycin Avermectin Nikkomycin
1990-2000	Spinosyn Tacrolimus

Table 1. Antibiotics since discovery of Streptomycin (adapted from Hopwood, 2007).

bacteria grown *in vitro.* Furthermore, by improving existing drugs such as glycylcylines and fluoroquinolones deriving from tetracyclines and quinolones, pharmaceutical industries aimed to stay "one step ahead" of resistant microorganisms. Although such an approach has been effective, it is becoming increasingly difficult to meet the needs of the community and to provide sufficient coverage for all emerging infectious agents (McDevitt and Rosenberg, 2001; Muzzi *et al.,* 2007).

To keep pace with microbial resistance, objective and target-directed strategies are needed to discover and develop new classes of antibiotics. In the light of the global threat outlined above, this chapter will overview emerging novel strategies with particular emphasis on bacteriophages as tools in the search for new and potent therapeutic agents from actinomycetes.

2. Genomics based approaches to drug discovery

Since early 2000s, information from completed genome sequences and genomic based technologies has been a driving force in antibiotic discovery resulting in new target identification of pathogens as well as in the enhancement of action studies of antimicrobial compounds. Exploitation of high-throughput automated DNA sequencing capabilities and genome sequences of microbial pathogens advanced rapidly producing full genome sequence results (e.g. *Enterococcus faecium* genome) (Amber, 2000; McDevitt and Rosenberg, 2001). In the past, antimicrobial studies were conducted on model microorganisms such as *E. coli* and *Bacillus subtilis,* however, with the new advances, research has become possible by directly using pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (McDevitt and Rosenberg, 2001; Payne *et al*., 2007). These developments have led to a shift in the discovery of novel vaccines and antimicrobials from the traditional empirical approach to a novel knowledge-based approach.

In conventional drug discovery, whole-cell screening approaches are adapted. This approach first identifies an antimicrobial compound and later seeks to establish the cellular target of that compound, and the vast majority of antibiotics that are currently used have this mode of action (e.g. targeting a limited number of proteins involved in critical cellular functions) (McDevitt and Rosenberg, 2001; Mills, 2003; Ricke *et al*., 2006). Whereas in the modern era of genome-driven and target-based approach a target gene is selected and its spectrum is identified. After it is validated for its role, cloned and sequenced, its corresponding protein product is expressed in an optimized expression system (e.g. *Pichia pastoris*, Baculovirus or *E. coli*). The target protein is then purified and screened against a large and diverse collection of low-molecular weight compounds in order to identify target inhibitors to investigate their potency, mechanism of inhibition and enzyme spectrum and selectivity (McDevitt and Rosenberg, 2001; Mills, 2003).

Increasing knowledge of bacterial diversity based on genomics and pangenomics now suggests that the way forward should be to focus discovery strategies on the identification of targets that are essential for the formation and persistence of an *in vivo* infection or in the expression of virulence factors *(*Muzzi *et al*., 2007).

Sequencing the entire genome of pathogens has revealed all of their open reading frames (ORFS), which can be utilized as selected targets in drug discovery. As summarized by McDevitt and Rosenberg (2001), there are several key criteria to be considered in target selection: "(1) the target should be present in a required spectrum of organisms; (2) it should be either absent in humans or, if present, it should be significantly different to allow confidence that selective inhibitors of the bacterial target over a human counterpart can be developed; (3) it should be essential for bacterial growth or viability under the conditions of the infection; (4) it should be expressed and relevant to the infection process; and (5) some aspects of its function should be understood to allow the relevant assays and high throughput screens to be developed".

One example was the use of peptide deformylase (PDF) as a protein target to facilitate discovery of a broad spectrum antibacterial drug (Mills, 2003). The protein is encoded by the *def* gene which is present in all pathogenic bacteria, but does not share a functionally equivalent gene in mammalian cells, which is one of the most sought after characteristics related to a drug candidate (McDevitt and Rosenberg, 2001; Yuan *et al*., 2001; Mills, 2003). This example was a good proof-of-principle illustration of the genomics-driven, target-based approach; starting with a conserved gene and leading to an antimicrobial compound (Clements *et al*., 2001; Mills, 2003).

Genome sequencing studies can also be utilized from the angle of drug producer microorganisms (Kurtböke, 2012). An example is the genome sequencing of *Salinispora tropica*, which has revealed its complex secondary metabolome as a rich source of drug-like molecules. Such a discovery has been a powerful interplay between genomic analysis and traditional natural product isolation studies (Udwary *et al*., 2007). Other examples that reveal the superior ability of actinobacteria to produce potent bioactive compounds facilitating discovery of novel bioactive compounds include genome sequences of *Streptomyces coelicolor* A3(2) (Bentley *et al*., 2002) and *S. avermitilis* (Ikeda *et al*., 2003).

3. Bacteriophages in chemotherapy

Therapeutic use of bacteriophages for the prevention and treatment of bacterial diseases, has been targeted since the discovery of phages in 1917 **by** Félix d'Hérelle. Following his discovery, he first attempted to use these against dysentery and since then, bacteriophages have been used to treat human infections as an alternative or a complement to antibiotic therapy (Hermoso *et al*., 2007). Particularly, from 1920s to 1950s, phage therapy has exploded and centres in the US, France and Georgia were established (Kütter and Sulakvelidze, 2005; Hermoso *et al*., 2007; Chanishvili, 2009), however, there have been limitations to antibacterial phage therapy that hamper its application as an antibiotic alternative. These have been summarized recently by Hermoso *et al*. (2007) as follows: (i) phages generally have narrow host range and only strongly lytic phage against bacterial strain infecting the patient, should be given to the patient; (ii) phages may not always remain lytic under the physiological conditions and bacteria can become resistant to phages after infection; (iii) phage preparations should be free of bacteria and their toxic components to meet clinical safety requirements, but sterilization of phage preparations could inactivate the phages; (iv) phages can be inactivated by a neutralizing antibody, and there is some risk of promoting allergic reactions to them; (v) the pharmacokinetics of phage treatments are more complicated than those of chemical drugs because of their self-replicating nature; (vi) phages might endow bacteria with toxic or antibacterial resistance genes.

Due to the above-listed limitations of bacteriophage therapy, bacteriophages might have more value as tools in drug discovery such as for target discovery and validation, assay development and compound design (Brown, 2004; Projan, 2004), and some of these exploitations are discussed below.

3.1 Bacterial virulence and injection mechanisms of bacteriophages

Efficient host infection relies on bacterial virulence factors being localized outside the producing cell where they are identically placed to interact with host defences and subvert host cells for the pathogen's benefit. Pathogenic bacteria have thus developed powerful

molecular strategies to deliver their virulence factors across the bacterial cell envelope as well as powerful mechanisms to adverse host cell plasma membrane (Cambronne and Roy, 2006; Filloux, 2009; 2011; Russell *et al*., 2011).

In Gram-negative bacteria, the cell envelopes have two hydrophobic inner and outer membranes with a hydrophilic space in between. The secreted hydrophobic molecules of proteins, enzymes or toxins have to travel through the hydrophobic environment of the membranes in an aqueous channel, or another type of conduit, that spans the cell envelope. These paths to the external medium are built by assembling macromolecular complexes, called secretion machines (Filloux, 2009; 2011) and they are distinguishable by the number and characteristics of the components such as types I, II and V secretion systems and they play important roles in the virulence of pathogens (Filloux *et al*., 2008; Leiman *et al*., 2009; Pukatzki *et al*. 2009; Bönemann *et al*., 2010; Schwarz *et al*., 2010).

In type VI secretion systems (T6SS) of Gram-negative bacteria the lack of an outer membrane channel for the T6SS might suggest an alternative delivery strategy such as local puncturing of the cell envelope to avoid cell lysis whilst allowing transient assembly of the secretion machine (Filloux, 2009; 2011). Filloux (2011) points out that the structural proteins of the T6SS are very similar to those that make up the injection machinery found in bacteriophages. Bacteriophages inject their DNA into bacterial cytosol and use the bacterium as a phage factory to replicate phage DNA. Bacterial cell envelope is perforated by bacteriophage puncturing device and its DNA is injected into bacterial cell via a tail tube. T6SS seems to use the same mechanism used by bacteriophages to inject their DNA into bacteria in which some components like the T6SS-specific exoproteins might have a similar tail-spike puncturing device of the T4 phage and might create a channel across the bacterial envelope which resembles the phage tail tube. T6SS translocation mechanism operate from the inside to the outside of the bacterial cell, and might be a mirror image of the phage translocation mechanism, which operates from outside to the inside of the bacterial cell Filloux (2011). Therefore, a sound understanding of bacteriophage injection and bacterial secretion systems might bring new insights to the development of effective therapeutic agents.

3.2 Bacteriophage-guided route to biodiscovery

Bacteriophages have evolved multiple strategies to interfere with bacterial growth. As a result, improved understanding of the bacteriophage-host interactions can also bring a new perspective to drug discovery (Young *et al*., 2000; Brown, 2004; Projan, 2004; Parisien *et al*., 2008). Examples include successful use of phage encoded lytic enzymes to destroy bacterial targets (Fishetti *et al*., 2003) and use of lysostaphin to achieve sterilization in an endocarditis model (Climo *et al*., 1998). Furthermore, in a novel approach, Liu *et al*. (2004) applied information deriving from phage genome to target discovery of gene products that inhibit pathogenic bacterium such as *Staphylococcus aureus*. They uncovered strategies used by bacteriophage to disable bacteria for design of a method, which uses key phage proteins to identify and validate vulnerable targets and exploit them in the identification of new antimicrobials.

Polysaccharide-specific phages were also suggested to treat encapsulated pathogenic bacteria since exolysaccharide production in bacteria involves biofilm formation and acts as a barrier to the penetration of therapeutic agents. Phages that can polymerize these substances

and/or kill the bacteria may potentially be useful for control of bacteria forming biofilms on medical devices (Hermoso *et al*., 2007). Protein antibiotics, which are the gene products of some small phages that do not produce endolysins, have also been shown to inhibit cell wall synthesis (Bernhardt *et al*., 2002). Genetic engineering of bacteriophages to carry toxic genes or proteins to produce cell death without lysis and hence avoiding the release of unwanted endotoxins has also been suggested (Westwater *et al*., 2003). Furthermore, Hagens *et al*. (2006) proposed a bacteriophage-based strategy to reduce effective doses of antibiotics during treatment for resensitization of antibiotic resistant pathogen via the presence of phage *in vivo*. In addition, it has been reported that phage host-cell lysis proteins, encoded by holins and amidases and elaborated late in the infection cycle, maintain their potent antibacterial activity when administered from outside cell (Loeffler *et al*., 2001; Schuch *et al*., 2002).

3.3 From bacteriophage genomics to drug discovery

Over evolutionary time, bacteriophages have developed unique proteins that arrest critical cellular processes to commit bacterial host metabolism to phage reproduction (Liu *et al*., 2004). Bacterial key metabolic processes can be shut off via inactivation of critical cellular proteins with these unique bacteriophage proteins, and host metabolism can be directed into the production of progeny phages. As an example; phages of *E. coli*, host physiology shuttoff is typically performed early during the phage lytic cycle by small phage-encoded proteins that target particularly vulnerable and accessible proteins involved in crucial host metabolic processes. Thus, Liu *et al*. (2004) using a high-throughput bacteriophage genomics strategy, exploited the concept of phage-mediated inhibition of bacterial growth to systematically identify antimicrobial phage-encoded polypeptides. They found that four proteins of the *Staphylococcus aureus* DNA replication machinery were targeted by a total of seven unrelated phage polypeptides leading to a superior approach to currently available antibiotics which only target topoisomerases. In some cases, sequence-unrelated polypeptides from different phages were found to target the same proteins in *S. aureus*, and such susceptibility might have uses in antimicrobial drug discovery.

All these developments including increased understanding of the mechanism of injection, beginning with adsorption to the host and ending with complete delivery of genomic material (McPartland and Rothman-Denes, 2009) are now paving the way towards recruitment of phages in the search for new antibiotics with previously unknown antibacterial mechanisms.

3.4 From endolysins to enzybiotics

Phages have different methods of progeny release from bacterial cells: filamentous phages are ejected from bacterial cell walls without destroying the host cell, whereas non-filamentous phages induce lysis through lytic enzymes. Phage lytic enzymes are highly evolved murein hydrolases to quickly destroy the cell wall of the host bacterium to release the progeny. Lysis is a result of abrupt damage to the bacterial cell wall by means of specific proteins and as stated by Hermoso *et al*. (2007) it can be completed in two different ways: (i) inhibition of peptidoglycan synthesis by a single protein or (ii) enzymatic cleavage of peptidoglycan by lysins or holin-lysin system.

Tailed phages achieve correctly-timed lysis by the consequtive use of endolysins and holins. Holins are small hydrophobic proteins that are encoded by the phage and inserted into cytoplasmic membrane to form membrane lesions or holes for endolysin passage. Whereas endolysins are phage-coded enzymes that break down bacterial peptidoglycan at the terminal stage of the phage reproduction cycle (Moak and Molineux, 2004; Loessner, 2005; Hermoso *et al*., 2007). Target specificity in endolysin studies reveal differences such as bifunctional enzyme of *Streptococcus agalactiae* phage with glycosidase and endopeptidase activities or muramidase activity of *Lactobacillus helveticus* phage (Loessner, 2005; Hermoso *et al*., 2007). However, most enzymes like amidases from phage that infect Gram-positive bacteria feature narrow lysis ranges, which can be genus-specific (*Streptomyces aureofaciens*) and even species-specific (*Clostridium perfringens*) (Loessner, 2005). Other examples include narrow specificity of endolysins only targeting *Clavibacter michiganensis* subspecies without affecting other bacteria in soil including closely related *Clavibacter* species (Wittmann *et al*., 2010).

Due to increasing antibiotic resistance, phage-derived lytic enzymes are now being exploited to control infections. In antibiotic resistant Gram-positive bacteria, it has been reported that even small quantities of purified recombinant lysin added externally lead to immediate lysis resulting in log-fold of death of the bacterial cells found on the mucosal surfaces and infected tissues. They have been suggested to make ideal antiinfectives due to lysin specificity for the pathogen that does not disturb the normal flora, the low chance of bacterial resistance towards lysins, and their ability to kill colonizing pathogens on mucosal surfaces illustrating a previously unavailable capacity (Hermoso *et al*., 2007, Fenton *et al*., 2010; Fishetti, 2010). These enzymes are suggested to particularly be useful to control antibiotic resistant Gram-positive pathogens. In this group of bacteria, lysins can make direct contact with their cell wall carbohydrates and peptidoglycan externally making them suitable candidates in clinical applications (Loessner, 2005; Hermoso *et al*., 2007).

Another example is *Mycobacterium,* phylogenetically related to Gram-positive bacteria but its cell envelope has a double-membrane structure similar to Gram-negative bacteria. Cell envelopes of mycobacteria contain peptidoglycan-arabinogalactan-mycolic acid complex (Sutcliffe, 2010). Mycobacteriophages must not only degrade the peptidoglycan layer but must also circumvent a mycolic acid-rich outer membrane covalently attached to the arabinogalactan-peptidoglycan complex. They utilize two lytic enzymes to produce lysis: (i) Lysin A that hydrolyzes peptidoglycan, and (ii) Lysin B, a novel mycolylarabinogalactan esterase, that cleaves the mycolylarabinogalactan bond to release free mycolic acids (Payne *et al*., 2009) and the study of phage ejection mechanisms in this group of bacteria might lead to the discovery of novel lytic systems and thus new antimicrobial agents.

Effective antimicrobial activity against Gram-positive bacterial pathogens including *Streptococcus pneumoniae* and *Bacillus anthracis* by exogenously applied phage-encoded endolysins has already been demonstrated. This approach has however, proved ineffective against Gram-negative bacteria since the outer membrane blocks access to the peptidoglycan targets (Fishetti, 2008). Due to their mycolic acid, rich outer membrane mycobacteria are likely to be similarly intractable to exogenously added endolysins. In order to overcome this resistance, a novel approach has been proposed by Payne *et al*. (2009) to render mycobacterial pathogens such as *M. tuberculosis* susceptible to endolysin treatment through co-treatment with LysA and LysB proteins.

In-depth understanding of the host-phage interaction and the full lytic-system is required to design effective biocontrol strategies using bacteriophage lysins. In this search, another rich source for mycobacterial phages might be the activated sludge systems where fascinating suborder, family, genus and species-specific host-phage interactions occur (Thomas *et al*., 2002). Recent genome sequencing of a *Tsukamurella* phage again isolated from an activated sludge system reveals a modular gene structure that shares some similarity with those of *Mycobacterium* phages (Petrovski *et al*., 2011). Accordingly, phylum level perspective and understanding of bacterial cell wall envelope architecture (Sutcliffe, 2010) with particular emphasis on monoderm and diderm bacteria, and translation of this understanding to phage lytic activity will advance current knowledge and contribute towards design and application of new phage-derived therapeutics. *Actinobacteria*-specific proteins, mainly specific for the *Corynebacterium*, *Mycobacterium* and *Nocardia* subgroups, have also been reported (Venture *et al*., 2007) and such specific proteins might have implications for the control of these pathogens. Mycetoma, a chronic granumatous infection persistent worldwide and endemic to tropical and subtropical regions, is another example (Linchon and Khachemoune, 2006) and among bacteria *Actinomadura* species reportedly cause the disease. However, in spite of trials in many different laboratories, phages specific to *Actinomadura* species were not reported until early 1990s (Long *et al*., 1993; Kurtböke *et al*., 1993b). Phages isolated towards different species of *Actinomadura* from organic mulches used in avocado plantations revealed that they belonged to *Siphoviridae* group of phages (Kurtböke *et al*., 1993b). Further studies on the *Actinodamura* phage and host-cell-wall interactions might shed light on the development of effective treatment strategies deriving from phage lytic activity on the pathogenic host.

Furthermore, metagenomics sequencing studies of uncultured viral populations have provided new insights into bacteriophage ecology. The cloning of phage lytic enzymes from uncultured viral DNA, and observations into colony lysis following exposure to inducing agent, revealed the value of viral metagenomes as potential sources of recombinant proteins with biotechnological value (Schmitz *et al*., 2010). Functional screens of viral metagenomes will inevitably provide a large source of recombinant proteins which might subsequently be used to treat infections resulting from difficult to control pathogens.

3.5 Prophage genomics

Prophage genomics has increased our understanding of the phage-bacterium interaction at the genetic level. Data deriving from these studies has also revealed genetic rules that underlie the arms race between the host bacterium and the infecting virus (Wagner and Waldor, 2002; Canchaya *et al*., 2003). Studies into non-pathogenic bacteria inhabiting varied but defined environments have also improved our understanding of the prophage contribution to the fitness increase of host bacterial cells. Even environmental and commensal bystander bacteria have been shown to be converted into toxin-producing ones via lysogenization (Chibani-Chennoufi *et al*., 2004).

Prophage genomics studies will possibly lead to discovery of important genes for the ecological adaptation of bacterial commensals and symbionts (Canchaya *et al*., 2003; Venture *et al*., 2007). Moreover, prophage genomics studies will provide further information on the expression of many lysogenic conversion genes (Canchaya *et al*., 2003) and all this information will then provide significant clues to be further exploited in drug discovery.

4. Natural products

Natural products have historically made significant contributions to the provision of new lead candidates in drug discovery programs (Newman and Cragg, 2004 a,b). Most characteristic features of the secondary metabolites are their incredible array of unique chemical structures and can be exploited as lead compounds, for chemical synthesis of new analogues or as templates, in the rational drug design studies. Their very frequent occurrence, versatile bioactivities and the rich structural and stereochemical attributes of natural products promote these compounds as valuable molecular scaffolds to explore their chemotherapeutic potential (Demain and Fang, 2000; Croteau *et al*., 2000; Firn and Jones, 2002). However, to continue to be competitive with other drug discovery methods, natural product research needs to continually improve the speed of the screening, isolation, and structure elucidation processes, as well addressing the suitability of screens for natural product extracts and dealing with issues involved with large-scale compound supply (Butler, 2004). Current alternative strategies include exploitation opportunities for drug discovery arising from an understanding of the mode of action of existing antibiotics. In this way, biochemical pathways or processes (e.g. peptidoglycan synthesis, tRNA synthesis, transcription and DNA replication) inhibited by antibiotics already in clinical use may contain key functions that represent unexploited targets for further drug discovery. Since most of these antibiotics are of natural product origin they might provide further clues in the search for their alternatives (Chopra *et al*., 2002).

4.1 Bioactive compounds from microbial resources

In industrial applications, microbial secondary metabolites are often defined as "low molecular mass products of secondary metabolism," which include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents and growth promoters (Demain and Fang, 2000; Bérdy, 2005; Bull, 2004; 2007; 2010) (Table 2 and 3).

Amino sugars	Glycopeptides	Phenazines	Pyrrolines
Anthocyanins	Glycosides	Phenoxazinones	Pyrrolizines
Anthraquinones	Hydroxylamines	Phthaldehydes	Quinolines
Aziridines	Indole derivatives	Piperazines	Quinones
Benzoquinones	Lactones	Polyacetylenes	Salicylates
Coumarines	Macrolides	Polyenes	Terpenoids
Diazines	Naphthalenes	Polypeptides	Tetracyclines
Epoxides	Naphthoquinones	Pyrazines	Tetronic acids
Ergot alkoloids	Nitriles	Pyridines	Triazines
Flavonoids	Nucleosides	Pyrones	Tropolones
Furans	Oligopeptides	Pyrroles	Vanillin
Glutarimides	Perylenes	Pyrrolidones	Zeaxanthin

Table 2. Examples of classes of organic compounds deriving from microbial secondary metabolites (adapted from Demain, 1981 and reproduced from Kurtböke, 2010a)

ACTH-like	Complement inhibition	Hemolytic	Leukemogenic
Anabolic	Convulsant	Hemostatic	Motility inhibition
Analeptic	Dermonecrotic	Herbicidal	Nephrotoxic
Anesthetic	Diabetogenic	Hormone releasing	Paralytic
Anorectic	Diuretic	Hypersensitizing	Parasympathomimetic
Anticoagulant	Edematous	Hypochloresterolemic	Photosensitizing
Antidepressive	Emetic	Hypoglycemic	Relaxant (smooth muscle)
Antihelminthic	Enzyme inhibitory	Hypolipidemic	Sedative
Anti-infective	Erythematous	Hypotensive	Serotonin antagonist
Anti-inflammatory	Estrogenic	Immunostimulating	Spasmolytic
Anti-parasitic	Coagulative (blood)	Hallucinogenic	Telecidal
Anti-spasmodic	Fertility enhancing	Inflammatory	Ulcerative
Carcinogenesis inhibition	Complement inhibition	Hemolytic	Vasodilatory
Coagulative (blood)	Hallucinogenic	Insecticidal	Anti-viral

Table 3. Pharmacological activities of microbial secondary metabolites (adapted from Demain, 1983 and reproduced from Kurtböke, 2010a)

However, existing antibiotics have mode of actions directed at a narrow spectrum of targets, principally cell wall, DNA and protein biosynthesis and so far multidrug resistance among bacterial pathogens has been largely due to a limited repertoire of antibacterial drugs that eradicate bacteria using a narrow range of mechanisms (Brown, 2004; Baltz, 2005; 2006a,b; 2008). Novel structural attributes are also required and only one new class of antibiotics has reached the clinic since 2001 (Ford *et al*., 2001). Currently, many novel microorganisms are being isolated from extreme biological niches, revealing their own chemical defence mechanisms. These naturally occurring organisms, together with recombinant organisms generated using combinatorial genetics and the availability of new chimeric metabolic pathways, might deliver an abundance of new compounds (Payne *et al*., 2007; Goodfellow 2010).

As advocates of natural product screening to search for novel antibacterial leads, Payne *et al.* (2007) adapted an alternative innovative approach with the belief that leads were not going to come from screening, but from alternative approaches. They reconsidered known antibacterial molecules to see whether they could improve their antibacterial and developmental properties and along these lines, they modified the pleuromutilin core structure in ways to bring three derivatives into clinical development. They also found lead molecules by screening a small, discrete library of compounds for antibacterial activity, which resulted in the discovery of a novel compound class capable of inhibiting bacterial DNA replication, and reached the developmental stage.

In the light of the above-mentioned advances, revisiting natural products with target-directed strategies might again provide us with novel and potent therapeutic agents.

4.2 Actinomycetes and drug discovery

Among the bacteria, the members of the order *Actinomycetales* have proved to be a particularly rich source of secondary metabolites with extensive industrial applications (Table 4).

Source	Bioactive secondary metabolites				
	Antibiotics		**Bioactive metabolites**		**Total bioactive metabolites**
	Total	With other activity	No antibiotic activity	Antibiotics plus other active compounds	
Bacteria	2900	780	900	1680	3800
Actinomycetes	8700	2400	1400	3800	10100
Fungi	4900	2300	3700	6000	8600
Total	16500	5500	6000	11500	22500

Table 4. Bioactive compounds of microbial origin (adapted from Bérdy, 2005 and reproduced from Kurtböke, 2010b).

In particular, the capacity of the members of the genus *Streptomyces* to produce commercially significant compounds, especially antibiotics, remains unsurpassed, possibly because of the extra-large DNA complement of these bacteria (Goodfellow and Williams, 1986; Kurtböke, 2010a; 2012). Members from this genus are even predicted to be the producers of many novel yet to be discovered bioactive compounds (Watve *et al*., 2001). As a result, selective isolation of previously undetected bioactive actinomycetes is one of the major targets of industrial microbiologists in the search for novel therapeutic agents (Bull *et al*., 2000; Bull and Stach, 2007; Goodfellow, 2010; Goodfellow and Fiedler, 2010; Kurtböke, 2003; 2010a).

The range of versatility of actinomycete metabolites is enormous and yields significant economic returns, yet, biodiscovery from these sources depends on the

i. detection and recovery of bioactive actinomycete fraction from previously unexplored environmental sources,
ii. effective assessment of their metabolites in defined targets (Goodfellow, 2010; Kurtböke, 2003; 2010a).

4.3 Bacteriophage-guided route to detection of rare actinomycetes

Chemical diversity of bioactive compounds, particularly from those rare and "yet to be discovered" actinomycetes is promising, however, detection of bioactive actinomycete taxa requires in-depth understanding of their true diversity and eco-physiology through which target-directed isolation strategies can be implemented (Bull *et al*., 2000; Bull, 2003; Kurtböke, 2012).

Isolation of bioactive rare actinomycete taxa requires highly specialised isolation techniques (Lazzarini *et al*., 2000; Kurtböke, 2003; Goodfellow 2010), and those employed range from the use of antibiotics to chemotaxis chambers, and excessive heat treatments (Hayakawa, 2003; Terekhova, 2003; Okazaki, 2003; Goodfellow 2010). In this context, bacteriophages have also proved to be useful tools in different applications, such as naturally-present indicators of under-represented or rare actinobacterial taxa in environmental samples; or as tools for deselecting unwanted taxa on the isolation plates in the process of target specific search for rare actinomycete taxa (Kurtböke, 2003; 2009; 2010b; 2011).

4.3.1 Actinophages as naturally-present indicators of rare actinomycetes in environmental samples

Presently, more than 50 rare actinomycete taxa are reported to be the producers of 2500 bioactive compounds (Bérdy, 2005), including several clinically important antibiotics such as vancomycin, erythromycin, tobramycin, apramycin, and spinosyns. However, these actinomycetes are not commonly cultured from natural substrates. Vancomycin producer *Amycolatopsis* sp. or spinosyn producer *Saccharopolyspora* sp. were found to be 4% and 3% abundant (Baltz, 2005).

Bacteriophages indicate presence of their host bacteria in an environmental sample and increased phage titre to detectable levels reflects the growth of indigenous host cells, and failure to do so reflects their absence from that source (Goyal, 1987). High densities of phages were reported in soils with conditions favourable for the host proliferation (Reanney and Marsh, 1973; Goyal *et al*., 1987). This ecological reality has been used to utilize bacteriophages as naturally-present indicators of under-represented or rare actinobacterial taxa in environmental samples (Williams *et al*., 1993; Kurtböke, 2003; Kurtböke, 2005; 2007; 2010b; 2011). Examples include detection of indicator phages towards actinomycetes including members of the genera *Saccharopolyspora* and *Salinispora* species (Kurtböke, 2009).

4.3.2 Exploitation of phages as deselection agents of unwanted taxa on isolation plates to recover rare actinomycetes from environmental samples

Direct analysis of rRNA gene sequences and birth of metagenomic studies showed that the vast majority of microorganisms present in the environment had not been captured by culture-dependent methods (Handelsman, 2004). Current advances such as microarrays

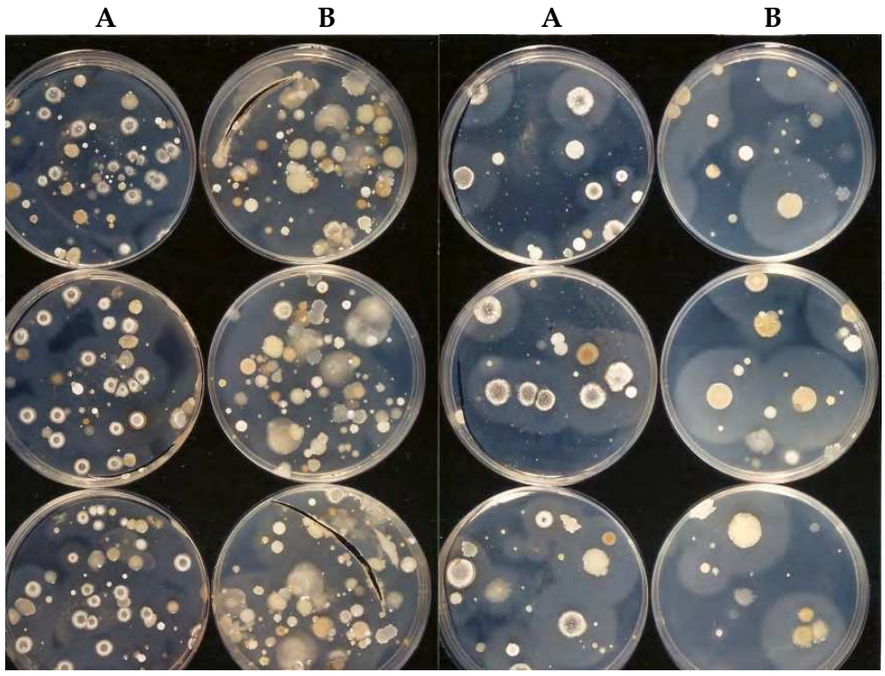

Fig. 1. Use of polyvalent streptomycete phage to reduce their numbers on isolation plates of a soil sample (A: without phage, B: with phage)

targeting the 16S rRNA gene of bacteria and archaea and the use of PhyloChips to identify specific members within a complex microbial community as well as targeting known functional gene markers to study functional gene diversity and activities of microorganisms in specific environment reveal true microbial diversity (Andersen *et al*., 2010). Functional gene arrays (GeoChips) have also been used to analyse microbial communities, and provide linkages of microbial genes/populations to ecosystem processes and functions (Andersen *et al*., 2010). Culturing representatives of these microorganisms with particular reference to previously explored environments such as those extreme and marine, has thus importance for biotechnological applications (Kennedy *et al*., 2007; Joint *et al*., 2010).

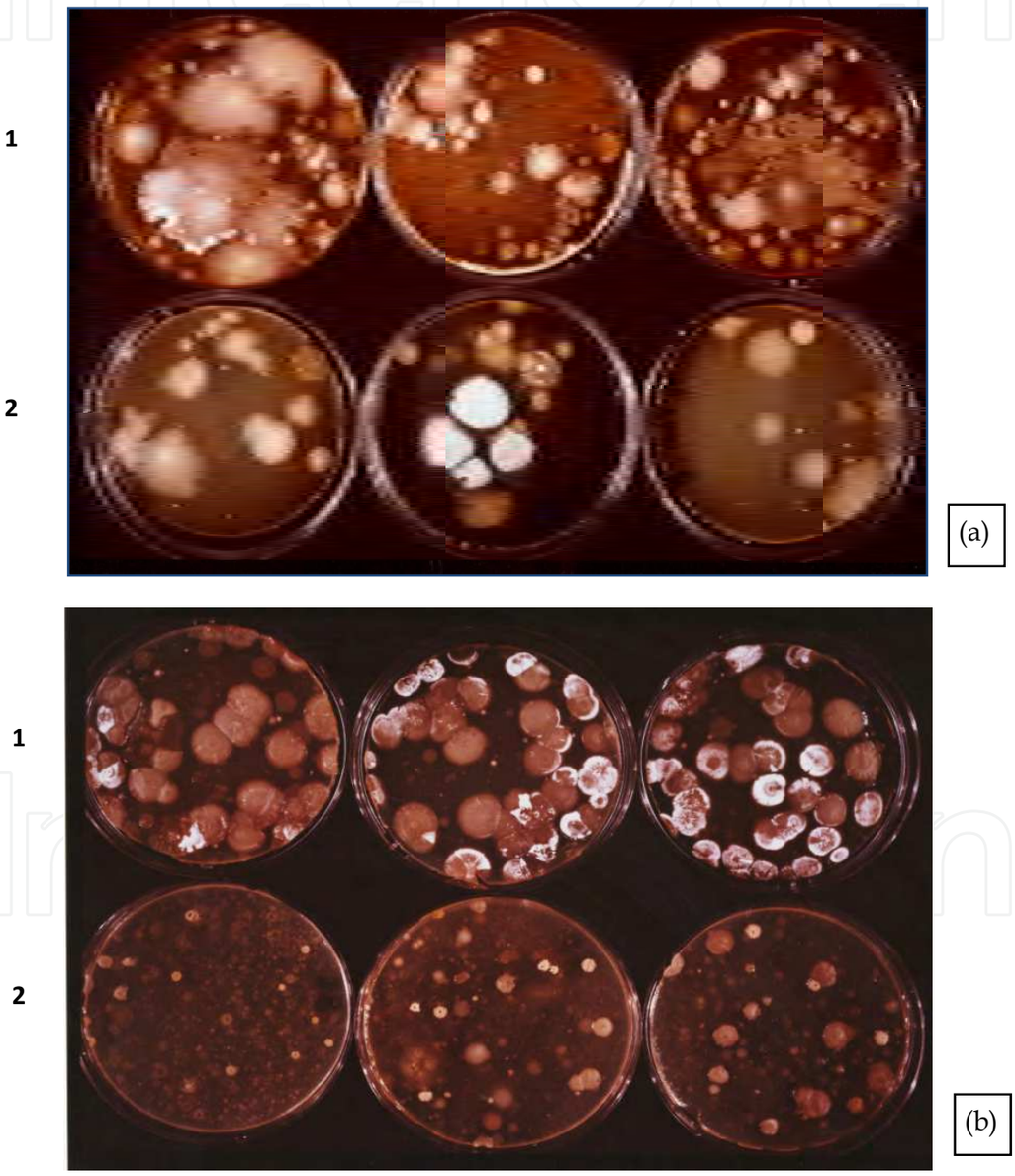

Fig. 2. Use of phage battery to reduce the numbers of (a) unwanted bacteria and (b) streptomycetes on ½ TSA plates to isolate rare actinomycetes. (1): Without phage, (2): With phage (reproduced from Kurtböke, 2010b)

Once information is generated on microbial diversity via above-listed molecular advances, phages can again be effective tools to remove unwanted taxa on the isolation plates in the process of target specific isolation of targeted taxa such as rare actinomycetes (Kurtböke *et al*., 1992; Kurtböke, 2003; Kurtböke, 2011). Examples include removal of smearing bacterial contaminants (e.g. as *Bacillus* species) rendering isolation of rare actinomycetes difficult from heated material on the isolation plates via phage battery (Kurtböke *et al*., 1993b; Kurtböke, 2003) (Figure 2 a,b).

Furthermore, layer by layer removal of unwanted soil taxa can also reveal bioactive fractions of the test sample under study (Kurtböke *et al*., 2002; Kurtböke and French, 2007). This fact is illustrated in Figure 1 where removal of streptomycete fraction of the sample reveals the presence of other bacterial taxa which are obviously susceptible to the antibiotic activity of streptomycetes (Kurtböke *et al*., 1992). This approach can particularly be useful in the detection of antimicrobial compound producing actinomycetes, even including novel streptomycetes in the samples, proved to be carrying antibiotic-resistant bacteria. It is a known fact that most studied environments can still yield novel members of bioactive genera (Williams *et al*., 1984) and revisiting these environments via the aid of indicator phages might render new bioactive species.

It is important to note that in-depth understanding of each sample's natural characteristics and its microfloral diversity is required for successful application of phage battery as a tool for selective isolation. In every different sample, a new set of bacteriophages suitable for the nature of the sample, has to be used to remove layers only to be present in that sample. Accordingly, new sets of phages obtained against contaminating background will be required for complete reduction in the numbers of background bacteria in each different sample (Kurtböke *et al*., 1992).

5. Conclusion

Bacteriophages can be powerful tools in the detection of bioactive actinomycetes and facilitate the discovery of novel bioactive compounds. They can offer more than we currently benefit from them if improved understanding of the host-phage ecology can be generated. Sound knowledge of microbial taxonomy is also a prerequisite for the effective use of bacteriophages in selective isolation procedures. Phage cross infectivity should also be interpreted carefully before they can be effectively exploited to select bioactive bacterial taxa (Kurtböke, 2011). In addition, current expansion of knowledge of phage and prophage genomics and phage infective mechanisms of host bacteria will provide a platform for the effective use of phages in biodiscovery.

Targeting host bacterial functional diversity, in which, certain metabolic activities might be triggered in a defined ecosystem following phage-mediated gene transfer might also offer clues for bioactivity (e.g. abolishment of rapamycin production as a consequence of phage insertion and its restoration upon the loss of the inserted phage by a second recombination (König *et al*., 1997)). An evaluation of the role of host-phage interactions in antibiotic production as well as in rendering antibiotics ineffective via lysogenation or prophage exertion will also further complement therapeutic success, and all this provides enough reason for the value of phages to be reconsidered in the post-genomic era (Kurtböke, 2011).

Current expertise of host receptor recognition by phages and the specificity of phage-derived lytic enzymes also needs to be developed further as well as an in-depth

understanding of the ecological and evolutionary reasons for monovalency and polyvalency (Kurtböke, 2011). Through such cumulative information, bacteriophages will gain increasing value as tools in drug discovery with their further use ranging from assay development to compound design (Brown, 2004; Projan, 2004).

6. In Memoriam

This chapter is dedicated to the memory of Professor Romano Locci (1937-2010), University of Milan and University of Udine, Italy.

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Bacteriophages have received attention as biological control agents since their discovery and recently their value as tools has been further emphasized in many different fields of microbiology. Particularly, in drug design and development programs, phage and prophage genomics provide the field with new insights. Bacteriophages reveals information on the organisms ranging from their biology to their applications in agriculture and medicine. Contributors address a variety of topics capturing information on advancing technologies in the field. The book starts with the biology and classification of bacteriophages with subsequent chapters addressing phage infections in industrial processes and their use as therapeutic or biocontrol agents. Microbiologists, biotechnologists, agricultural, biomedical and sanitary engineers will find Bacteriophages invaluable as a solid resource and reference book.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

D.I. Kurtböke (2012). A Phage-Guided Route to Discovery of Bioactive Rare Actinomycetes, Bacteriophages, Dr. Ipek Kurtboke (Ed.), ISBN: 978-953-51-0272-4, InTech, Available from: http://www.intechopen.com/books/bacteriophages/a-phage-guided-route-to-discovery-of-bioactive-rare-actinomycetes

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