Bacteriophage 1:4, 195-197; July–December 2011; © 2011 Landes Bioscience

Phage as a source of antibacterial genes
Multiple inhibitory products encoded by Rhodococcus phage YF1

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Key words: multidrug resistance, bactericidal proteins, antibacterial drug discovery, target identification, phage YF1

Abbreviations: AIDS, acquired immune deficiency syndrome; ORF, open reading frame

Bacteriophage-encoded proteins which inhibit or modify cellular components may contribute to antibacterial drug discovery by allowing the identification of novel targets. Given their abundance and diversity, phages may have various strategies in host inhibition and therefore may possess a variety of such proteins. Using *Rhodococcus equi* and phage YF1, we show that a single phage possesses numerous genes that inhibit the host when introduced into the host on a plasmid. These genes mostly encode proteins of unknown function, confirming the potential that this approach may have in providing new antibacterial targets.

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Bacteriophages encode early proteins that may alter or inactivate indispensable host proteins upon infection.[1] In an effort to combat multidrug resistance in bacterial pathogens, these host proteins might serve as novel targets in antibacterial drug discovery. This approach was demonstrated by the work of Liu et al.[2] who identified both phage polypeptides and their cellular targets in *Staphylococcus aureus*, as well as small molecules which could bind the cellular targets and inhibit the pathogen. As interactions of phage proteins with their targets are expected to be essential for phages to engage in their lytic cycle, effective antibacterials might be discovered through this method. Considering phage abundance in the environment and their tremendous diversity,[3] there should be an existent phage and therefore an existent strategy to combat any given bacterial pathogen, so exploration of the strategy reported has yet to meet its full potential. In this work we focused on *Rhodococcus equi*,[4] a Gram-positive, pulmonary, intracellular pathogen of foals and AIDS patients, as a model to demonstrate that a phage infecting this pathogen can be used to elucidate a number of potential targets for the development of antimicrobial products. Although *R. equi* is a rarely encountered human opportunistic pathogen, its close relation to significant pathogens from genera such as Mycobacterium and Corynebacterium and the emergence of multidrug resistant strains[5] place this species in an ideal framework for antimicrobial drug discovery.

As the rhodococci are ubiquitous soil saprophytes, we first isolated from soil a phage plaquing on *R. equi* ATCC 14887. On a lawn of the host bacteria, a single soil sample incubated in rich broth yielded plaques of various sizes and morphologies, one of which was plaque purified and now named YF1. Observation of purified, negatively-stained YF1 particles under the electron microscope revealed a head-and-tail structure of the *Siphoviridae* family (**Fig. 1A**). Besides *R. equi*, the phage was capable of plaquing on *Rhodococcus erythropolis* ATCC 4277, *Rhodococcus rhodochrous* Ri8 and *Rhodococcus opacus* HL PM-1 at efficiencies of plaquing of 1.0, 0.8 and 0.1, respectively, but not on *Gordonia australis* A554, *Gordonia rubropertincta* ATCC 25593, *Gordonia desulphuricans* NB4, *Nocardia mexicana* IFO 3927, *Mycobacterium smegmatis* mc^{2}155 or *Mycobacterium parafortuitum* IFM 0490. The YF1 genome size was estimated to be 55 kb using pulsed-field electrophoresis (**Fig. 1B**).

Two genomic libraries of YF1 were constructed in *Escherichia coli* by shotgun-cloning of *Bgl*II and *Pst*I digests separately into the *E. coli*-Rhodococcus shuttle vector pDA71,[6] which is a fusion of *E. coli* vector pEcoR251 and the replicon from a Rhodococcus phage Q4, and has a low copy-number in Rhodococcus. The polylinker of pDA71 is flanked on one end with the phage λ P_R promoter. Enzymes *Bgl*II and *Pst*I have recognition sites in this polylinker and also generated many YF1 DNA fragments in the size range of 1–6 kb upon complete digestion (**Fig. 1C**); thus they were deemed suitable for library construction. The first library consisted of 140 clones of *Bgl*II digests, with an average insert size per clone of 2.5 kb and covering 99% of the genome, calculated using an equation described by Clarke and Carbon.[7] Upon screening 20 clones, 85% of them had detectable inserts. The second library consisted of 95 clones of *Pst*I digests, with an average insert size per clone of 2.7 kb and also covering 99% of the genome. Upon screening 20 of these clones, 80% of them

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Submitted: 05/25/11; Revised: 08/12/11; Accepted: 08/13/11
http://dx.doi.org/10.4161/bact.1.4.17746

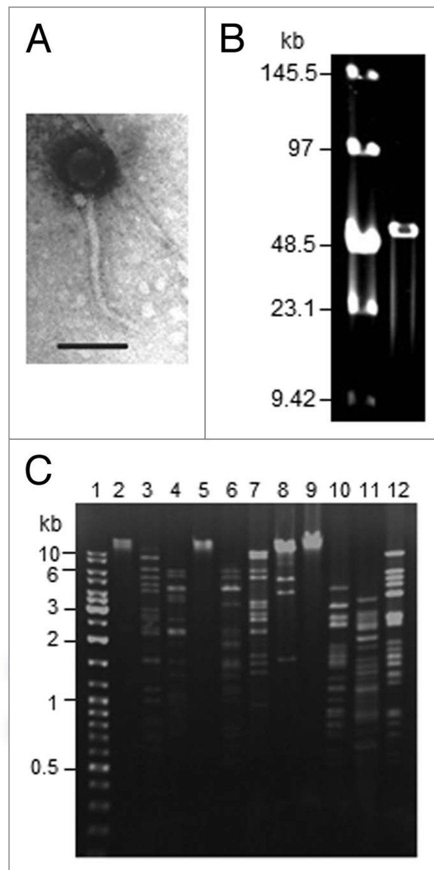

Figure 1. Phage YF1 characteristics. (A) Electron micrograph of negatively stained phage YF1 (bar = 100 nm), (B) pulsed-field gel electrophoresis of YF1 genome and (C) restriction digests of YF1 genomic DNA. Lane 1, molecular weight marker; 2, undigested control; 3, *Hind*III; 4, *Bgl*II; 5, *Bam*HI; 6, *Bcl*I; 7, *Pst*I; 8, *Nsi*I; 9, *Bmy*I; 10, *Sfu*I; 11, *Acc*I; 12, *Cla*I.

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had detectable inserts. Twenty randomly-selected clones from each library, representing roughly 60% coverage of the YF1 genome in the case of the *Bgl*II library and 63% in the case of the *Pst*I library, were screened for an inhibitory phenotype by transforming them into *R. equi* in triplicate. Six clones from the *Bgl*II library and nine clones from the *Pst*I library were shown to have transformation efficiencies of at least two orders of magnitude less than that of vector-only control (2.6 x 10^4/μg DNA), suggesting inhibition associated with the cloned phage DNA. Of these clones, four from the *Bgl*II library and three from the *Pst*I library were selected for further study. Where possible, regions of DNA with no apparent contribution to the inhibitory phenotype were removed by restriction digestion and subcloning, although in some cases the phenotype was not narrowed down to a single open reading frame (ORF). Following sequencing of these DNA (from ten subclones in total), 13 ORFs were predicted to possess a *R. equi* inhibition phenotype. Moreover, ten of these did not possess similarities that enabled function prediction using BLASTn against the nucleotide collection database or BLASTx against the non-redundant protein sequence database (**Table 1**). A myriad of novel bactericidal or bacteriostatic activities may be associated with products encoded by these genes with unknown functions, and the focus of future studies should be to search for their respective cellular targets. When the predicted phage protein sequences were searched for functional domains, repeats or signatures against the InterPro database (www.ebi.ac.uk/interpro/), the only predictions that could be made were transmembrane domains in clones pYF1B7A and pYF1B19A. It is difficult to assume their function from these data alone.

It is important to note a few points here. None of the clones in this study are inhibitory against *E. coli*, as the library was constructed in this host. This means either that the activities of the products encoded by the clones are specific toward Rhodococcus or potentially other closely-related Gram-positive bacteria, or that those genes were simply not expressed in *E. coli*. Flanking the polylinker on one end in pDA71, the phage λ P_R promoter drives expression of cloned genes in *E. coli*. This promoter probably functions sub-optimally in Rhodococcus; even if it did drive transcription in this host, we expect its level to be low. No known promoter exits on the opposite side of the polylinker. As the phage DNA fragments were ligated into the vector in either one of two possible orientations, most of expression of these phage genes in Rhodococcus were probably off their native phage promoters. It is also important to caution that not all of these genes may necessarily encode products which are designed to inactivate or modify essential cellular enzymes during phage infection. For example, the thymidylate synthase complementing protein,[8] which is potentially encoded by one of the inhibitory YF1 genes (**Table 1**), may simply be interfering with the balance of cellular enzymes in the pathway for thymidine production. Nucleotide production (specifically, folate biosynthesis), however, is a well-known pathway which is targeted by the sulphonamide class of antibiotics and may represent a weakness in bacteria. Coincidentally, the thymidylate synthase complementing protein has been investigated as a drug target as its distribution is limited mainly within eubacteria and archaea.[9]

Spread of multidrug resistance in pathogenic bacteria has not been countered by the discovery and development of new classes of antibiotics. As natural killers of bacteria, phages have the potential to make important contributions in several ways: (1) through phage therapy using phage particles as therapeutic agents,[10] (2) through the use of phage lysins or other phage-encoded bacterial killing agents,[11] and (3) indirectly, through the use of phage to identify cellular components targeted by phage proteins that have the potential to serve as targets for antimicrobial design. Each of these options relies on the abundance and diversity of phages in nature; for identification of novel targets, it is also desirable that multiple targets be identified from a single virus. Although only a small portion of the identified YF1 genes may encode products that specifically target indispensable host components, our work shows that a mere single phage that was readily isolated from the environment, possessing a relatively small genome of 55 kb, contains a wealth of genes that are inhibitory upon introduction into a bacterial host. Genomes of phages for any pathogen of interest may be screened in a similar fashion. These may include methicillin-resistant *S. aureus* and multidrug-resistant *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* to name a

Table 1. Summary of YF1 DNA inhibiting *R. equi*

Library	Plasmid	Insert size (bp)	No. predicted ORFs[a]	Prediction of potentially encoded product[b]	Accession number
***Bgl*II**	pYF1B7A	670	1	None	DQ981382
	pYF1B7B	790	1	None	DQ981383
	pYF1B8	920	2	*Thymidylate synthase* complementing protein[c]	DQ981384[d]
	pYF1B10	610	2	None	DQ981385
	pYF1B19A	710	2	None	DQ981386
	pYF1B19B	780	1	None	DQ981387
***Pst*I**	pYF1P8A	800	1	Prohead protease	DQ981388
	pYF1P8B	800	1	Prohead protease	DQ981389[d]
	pYF1P14	390	1	None	DQ981390
	pYF1P16B	800	1	None	DQ981392

[a]Where two ORFs are present, the inhibitory phenotype was not narrowed down to a single ORF. [b]Potentially encoded products were predicted by comparing YF1 sequences to those in the database. "None" indicates absence of aligning sequences encoding products of known function with an E-value of $< 10^{-4}$. [c]Of two predicted ORFs, only one of them had a significant similarity match in the database. [d]These have similarity matches to sequences of unknown function in the environmental samples database.

few pathogens whose global spread is a major threat to human health. To continue this phage-mediated approach by identifying cellular targets and small molecules with similar activities is not a trivial task. As Liu et al. have shown, these involve various biochemical assays and a high-throughput screening of chemical libraries. Given its potential, however, this approach is one that could be pursued if we are to turn around what seems to be a losing battle against superbugs.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

This work was supported by the National Research Foundation and the Medical Research Council of South Africa.

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