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

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 demonstrate that a phage infecting this pathogen can

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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As the rhodocci 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 R18 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 mc²155 or Mycobacterium parafloratum 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 BglII and PstI digests separately into the E. coli–Rhodococcus shuttle vector pDA71,4 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 Pₐ promoter. Enzymes BglII and PstI 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 BglII 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 PstI 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
Bacteriophage had detectable inserts. Twenty randomly-selected clones from each library, representing roughly 60% coverage of the YF1 genome in the case of the BglII library and 63% in the case of the PstI library, were screened for an inhibitory phenotype by transforming them into R. equi in triplicate. Six clones from the BglII library and nine clones from the PstI library were shown to have transformation efficiencies of at least two orders of magnitude less than that of vector-only control (2.6 x 10^3/μg DNA), suggesting inhibition associated with the cloned phage DNA. Of these clones, four from the BglII library and three from the PstI 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 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 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 Pr 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, 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 euabacteria and archaea.

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, (2) through the use of phage lysins or other phage-encoded bacterial killing agents, 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 not been countered by the discovery and development of new classes of antibiotics.
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.

References
1. Sau S, Chattoraj P, Ganguly T, Chanda PK, Mandal NC. Inactivation of indispensable bacterial proteins by early proteins of bacteriophages: implication in antibacterial drug discovery. Curr Protein Pept Sci 2008; 9:284-90; PMID:18537683; http://dx.doi.org/10.2174/138920308784533970
2. Liu J, Dehbi M, Moeck G, Arhin F, Bauda P, Bergeron D, et al. Antimicrobial drug discovery through bacteriophage genomics. Nat Biotechnol 2004; 22:185-91; PMID:14716317; http://dx.doi.org/10.1038/nbt932
3. Clokie MRJ, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage 2011; 1:31-45; PMID:21687533; http://dx.doi.org/10.4161/bact.1.1.14942
4. von Bargen K, Haas A. Molecular and infection biology of the horse pathogen Rhodococcus equi. FEMS Microbiol Rev 2009; 33:870-91; PMID:19453748; http://dx.doi.org/10.1111/j.1574-6976.2009.00181.x
5. Corti M, Palmero D, Ejiguchi K. Respiratory infections in immunocompromized patients. Curr Opin Pulm Med 2009; 15:209-17; PMID:19276812; http://dx.doi.org/10.1097/MCP.0b013e32830fbbd
6. Quan S, Dubbs ER. Nocardioform arsenic resistance plasmid characterization and improved Rhodococcus cloning vectors. Plasmid 1993; 29:74-9; PMID:8441772; http://dx.doi.org/10.1006/plas.1993.1010
7. Clarke L, Carbon J. A colony bank containing synthetic Col El hybrid plasmids representative of the entire E. coli genome. Cell 1976; 9:91-9; PMID:788919; http://dx.doi.org/10.1016/0092-8674(76)90055-6
8. Myllykallio H, Lipowski G, Ledac D, Filee J, Forterre P, Liebl U. An alternative flavin-dependent mechanism for thymidylate synthesis. Science 2002; 297:105-7; PMID:12029065; http://dx.doi.org/10.1126/science.1072113
9. Mathews II, Deacon AM, Canaves JM, McMullan D, Lesley SA, Agarwalla S, et al. Functional analysis of substrate and cofactor complex structures of a thymidylate synthase-complementing protein. Structure 2003; 11:677-90; PMID:12791256; http://dx.doi.org/10.1016/S0969-2126(03)00097-2
10. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage 2011; 1:66-85; http://dx.doi.org/10.4161/bact.1.2.15845
11. Hanlon GW. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. Int J Antimicrob Agents 2007; 30:118-28; PMID:17566713; http://dx.doi.org/10.1016/j.ijantimicag.2007.04.006

Table 1. Summary of YF1 DNA inhibiting R. equi

| Library | Plasmid | Insert size (bp) | No. predicted ORFs | Prediction of potentially encoded product | Accession number |
|---------|---------|-----------------|-------------------|---------------------------------------------|-----------------|
| BglII   | pYF1B7A | 670             | 1                 | None                                        | DQ981382        |
|         | pYF1B7B | 790             | 1                 | None                                        | DQ981383        |
|         | pYF1B8  | 920             | 2                 | Thymidylate synthase complementing protein   | DQ981384d       |
|         | pYF1B10 | 610             | 2                 | None                                        | DQ981385        |
|         | pYF1B19A| 710             | 2                 | None                                        | DQ981386        |
|         | pYF1B19B| 780             | 1                 | None                                        | DQ981387        |
|         | pYF1P8A | 800             | 1                 | Prohead protease                            | DQ981388        |
|         | pYF1P8B | 800             | 1                 | Prohead protease                            | DQ981389d       |
|         | pYF1P14 | 390             | 1                 | None                                        | DQ981390        |
|         | pYF1P16B| 800             | 1                 | None                                        | DQ981392        |

*Where two ORFs are present, the inhibitory phenotype was not narrowed down to a single ORF. 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. Of two predicted ORFs, only one of them had a significant similarity match in the database. These have similarity matches to sequences of unknown function in the environmental samples database.