Abstract

Over billions of years of evolution, viruses of eubacteria, bacteriophages, have developed unique proteins enabling them to arrest or inhibit critical bacterial cellular processes to augment phage reproduction. To address concerns of worldwide increase in antibiotic resistance, we have exploited the mechanisms of bacterial growth inhibition employed by phages to identify novel targets for antibiotic discovery. Using high-throughput bacteriophage genomics and functional genomics, we sequenced genomes of 43 phages that infect three pathogens and screened for open reading frames (ORFs) that inhibit bacterial growth. These phage-derived inhibitory ORFs were used as baits to systematically identify their interacting partners in bacteria. A variety of protein targets, biologically validated by phages, have been discovered. HTS campaigns were carried out against these targets. Small molecule leads with antibacterial activity were identified. By taking advantage of what we have learned from Nature, our technology platform plays an important role in the battle against antibiotic resistance.

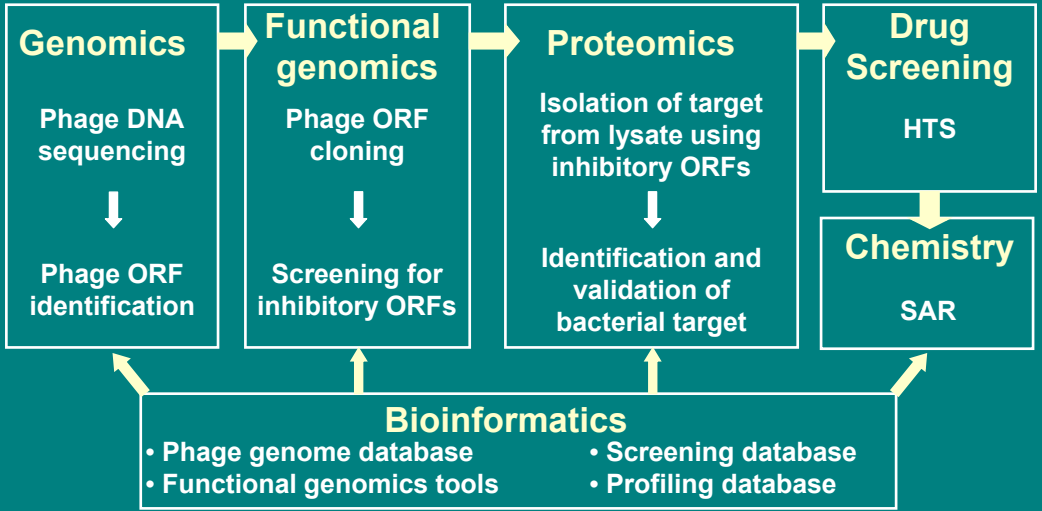
Current Approaches to Antimicrobial Drug Discovery

- Screen candidate molecules in whole-cell-based assays for antibacterial activity
- Synthesize analogs of known drugs or candidates with improved characteristics
- Discover novel compounds against known targets with established mode of action
- **Target-based drug discovery on phage-selected targets: PhageTech approach**

Key advantages of PhageTech's proprietary drug discovery platform:

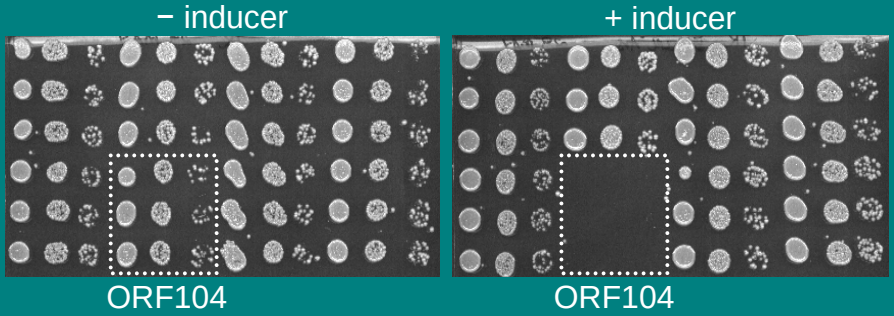
- speed of analysis of small phage genomes
- access to diversified targets
- evolutionary validation of the bacterial target

PhageTech's Antimicrobial Discovery Tools



Screening for Antimicrobial Phage ORFs

Transform *S. aureus* with phage ORFs → Dot clones onto medium +/- inducer → Identify antimicrobial ORFs under induced conditions



- Induction of expression of phage ORF104 inhibits *S. aureus* growth

Success of PhageTech's Genomics and Functional Genomics Programs

Bacterial pathogen	Phages collected	Genomes sequenced	ORFs screened	Inhibitor families
S. aureus	150	27	964	31
S. pneumoniae	50	8	264	5
P. aeruginosa	70	11	500	13
Total	**270**	**46**	**1728**	**49**

ORF Affinity Approach to Target ID

Affinity chromatography of bacterial lysate over immobilized antimicrobial phage ORF

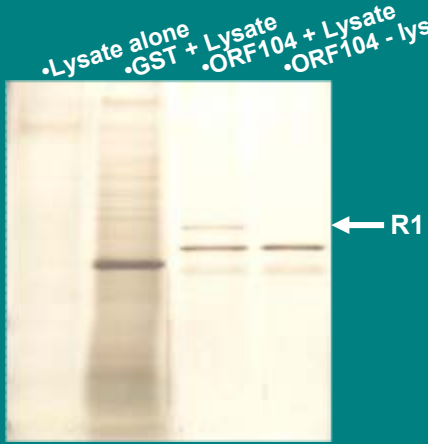
Tryptic peptide mapping, mass spectrometry of eluted bacterial proteins

Target identification

Target validation

- Confirm ORF-target interaction
- Confirm target essentiality
- Determine target function

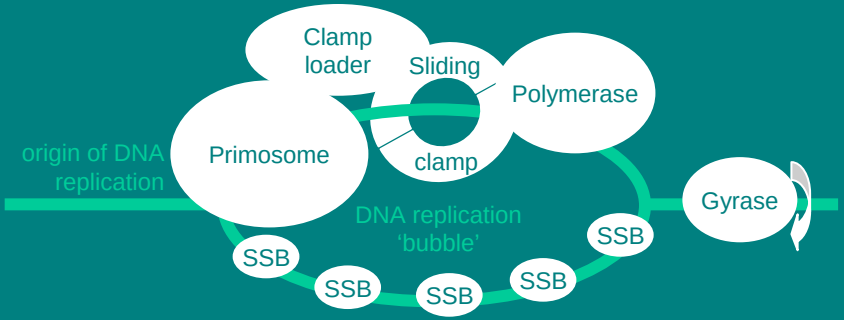
S. aureus R1 is the Target of ORF104



- *S. aureus* target R1 was isolated using immobilized antimicrobial phage ORF104
- R1 was identified as a target within the DNA synthesis pathway of *S. aureus*

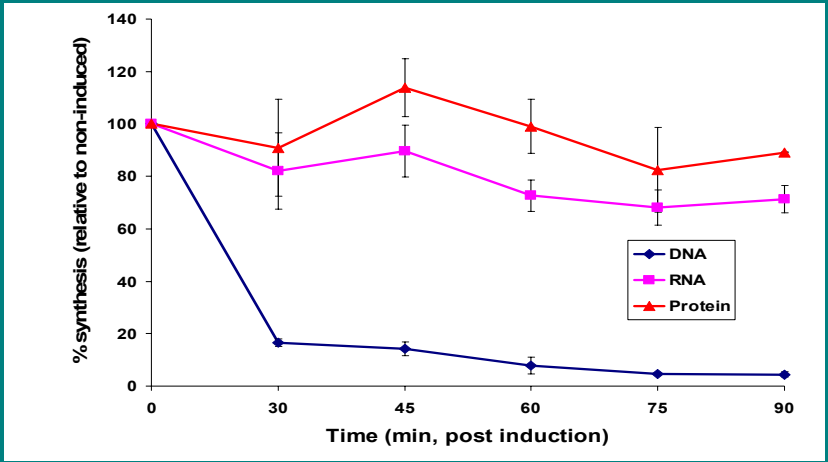
R1 is Implicated in DNA Replication

Proteins and protein sub-assemblies involved in replication:



Hypothesis: inhibition of R1 activity with ORF104 should inhibit DNA replication

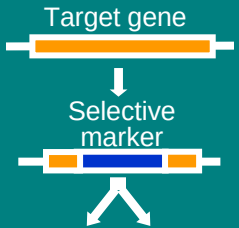
ORF104 Inhibits ^{3}H-thymidine Incorporation



- Expression of ORF104 rapidly and selectively inhibits DNA synthesis in *S. aureus*

Essentiality Analysis of *S. aureus* Genes

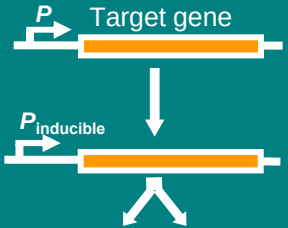
Gene inactivation/ replacement



Growth: target not essential

No growth: suggests target is essential

Regulated gene expression



Growth *without* inducer: target not essential

Growth only *with* inducer: suggests target is essential

The *R1* Gene is Essential in *S. aureus*

Gene inactivation/ replacement:

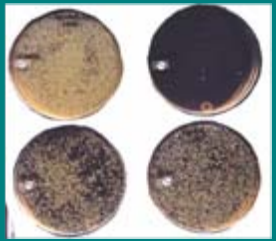
- no viable clones obtained
- suggests that *R1* is an essential gene

Regulated gene Expression:

+ inducer **- inducer**

***R1* $P_{inducible}$**

Wild-type *S. aureus*



- growth of *R1* $P_{inducible}$ strain requires inducer
- confirms essentiality of *R1* gene in *S. aureus*

S. aureus DNA and RNA Synthesis Targets Identified by Antimicrobial Phage ORFs

Antimicrobial ORF family	ORF size (aa)	Target (biosynthetic pathway of target)	Essentiality of target
ORF104 ORF16	52 297	R1 (DNA)	Essential
ORF25 ORF168 ORF240	58 74 58	R2 (DNA)	Essential
ORF78	71	R9 (DNA)	Essential
ORF67	198	R12 (RNA)	Essential

Screening Strategy

Small molecule compounds

↓ Proprietary HT screens:
- ORF-target interaction
- enzymatic activity of target

Actives

↓ Confirmatory, counter screening (n=2; 20 μM)

Confirmed actives

↓ Dose response testing (n=4; 0.3-100 μM)

Hits

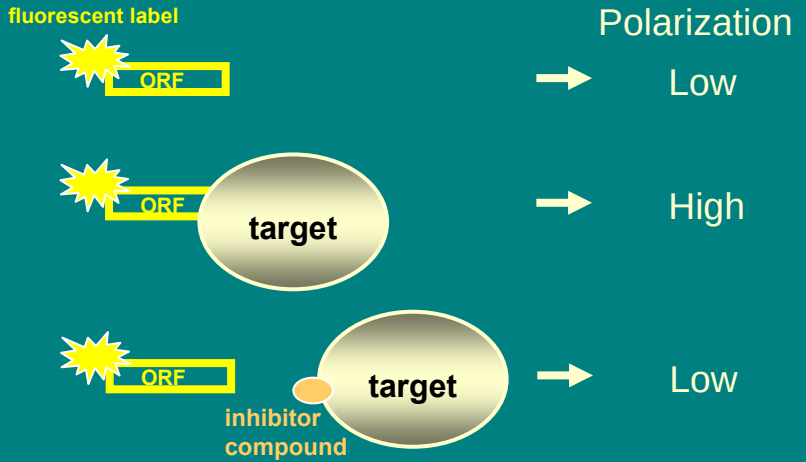
↓ *In vitro* functional assay, if available

Confirmed hits

↓ Susceptibility testing, compound profiling

Lead

FP Assay for ORF104-R1 Interaction



HTS FP Assay for ORF104-R1 Inhibitors

- Despite a high FP value (149 mP) for the free ORF tracer (7 kDa), R1 (39 kDa) increased the polarization of the tracer (to 223 mP) and permitted assay development
- Reproducibility at pilot scale was acceptable
- 125, 000 compounds were screened at 20 μM in a 20 μL 384-well FP assay
- Assay performance:
  - Z': 0.6
  - Z: 0.5
  - $FP_{compounds}$: 223 mP +/- 11.2%
  - $FP_{+\ control}$: 149 mP +/- 3.5%
  - $FP_{-\ control}$: 223 mP +/- 5.5%

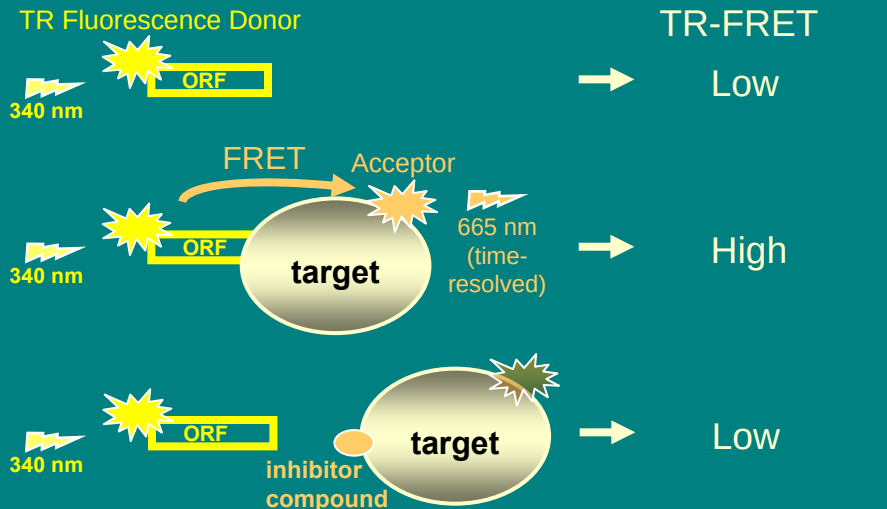
  } ΔFP = 74 mP

  - Throughput: 7500 cpds/day (Tecan Genesis/TeMo + 1 operator)
  - Cost: $0.05/well

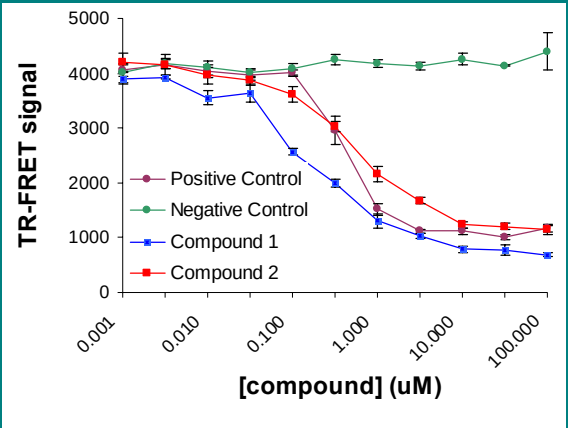
Hit Compounds Identified Against *S. aureus* Targets

Target	Assay	Library size	Actives	Confirmed actives	Hits $IC_{50} \leq 10$ µM	Confirmed Hits MIC ≤ 16 µg/ml
R1 (DNA)	FP	125 K	1311	156	73	16
R9 (DNA)	TR-FRET	125 K	932	464	90	24
R12 (RNA)	TCA	125 K	830	153	73	36

TR-FRET Assay for ORF-Target Interaction



Dose-Response Testing of Confirmed Actives Against ORF104-R1 by TR-FRET



- Several compounds with IC_{50}≤10 μM were identified in the TR-FRET assay

Mechanism of Action Studies Using Target Titrating System

- Engineer *S. aureus* strain to express target under control of inducible promoter
- Test susceptibility to small molecule compounds with increasing concentrations of inducer (IPTG)
- Use antibiotics against known targets as controls

- Data suggest that compounds kill bacteria *via* intended target
- Confirmed hits are now the focus of SAR / chemistry programs

Summary

- PhageTech has sequenced the genomes of 47 phages of *S. aureus, S. pneumoniae,* and *P. aeruginosa*
- 49 families of phage-derived antimicrobial ORFs were identified
- Biologically validated targets, including those essential for DNA and RNA synthesis in *S. aureus*, have been discovered
- Proprietary ORF-target binding assays and enzymatic assays have been developed around these targets
- Diverse libraries of small molecules are being screened for inhibitors and selected compounds are being optimized
- Target titration systems have been developed and have been used to demonstrate mechanism of action
- Some lead compounds show promising profiles against both Gram-positive and Gram-negative bacteria (including drug resistant organisms) and will be evaluated in animal models