SAND2016-3874PE

Designing CRISPR Antimicrobials

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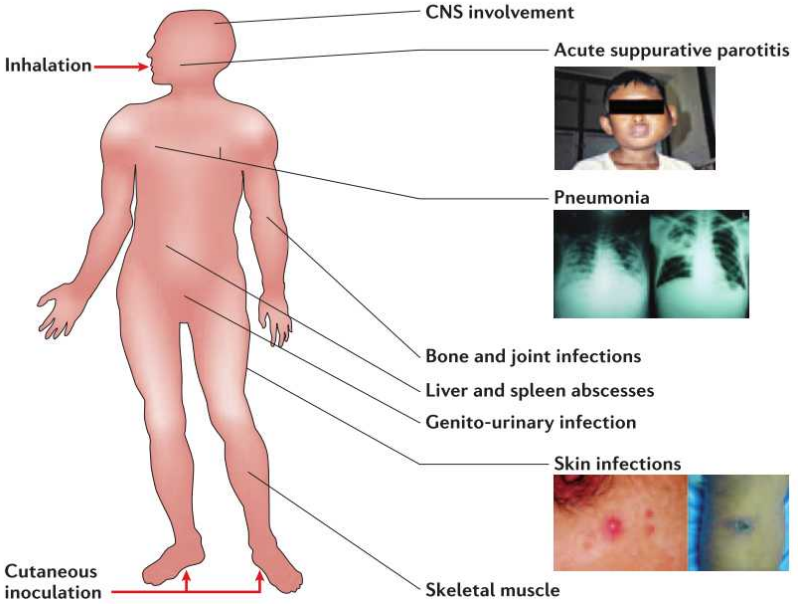
Berkeley Visit

Burkholderia pseudomallei

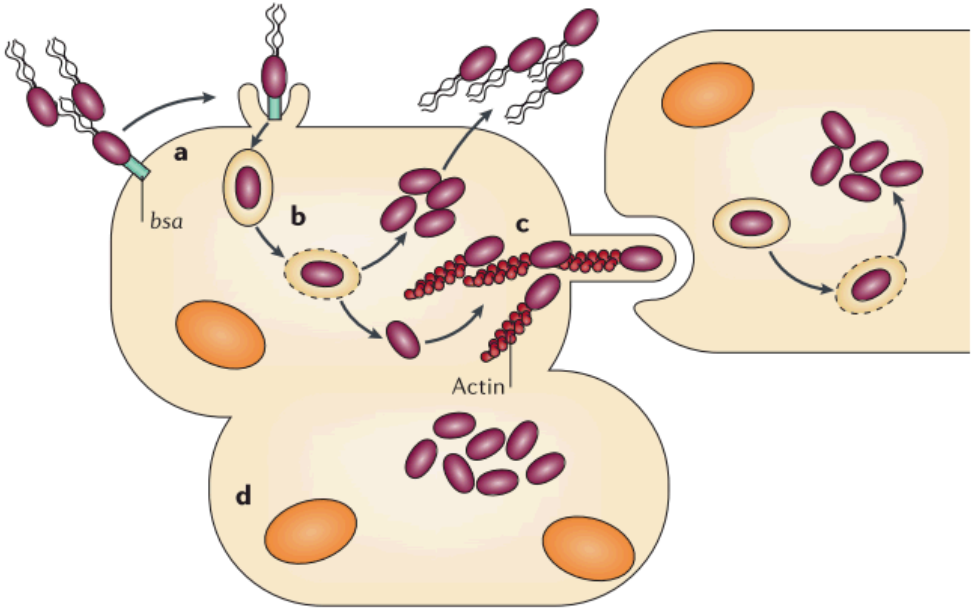
- *Burkholderia pseudomallei* is a highly drug resistant, intracellular gram-negative bacterium
- Common in Southeast Asia and Northern Australia
  - 20-50% mortality rate
- Infection is acquired by inoculation, inhalation, and aspiration
- Causes melioidosis
  - Pneumonia
  - Bone pain
  - Abscesses
  - Brainstem encephalitis

- ***Goal: Develop novel treatment strategies***



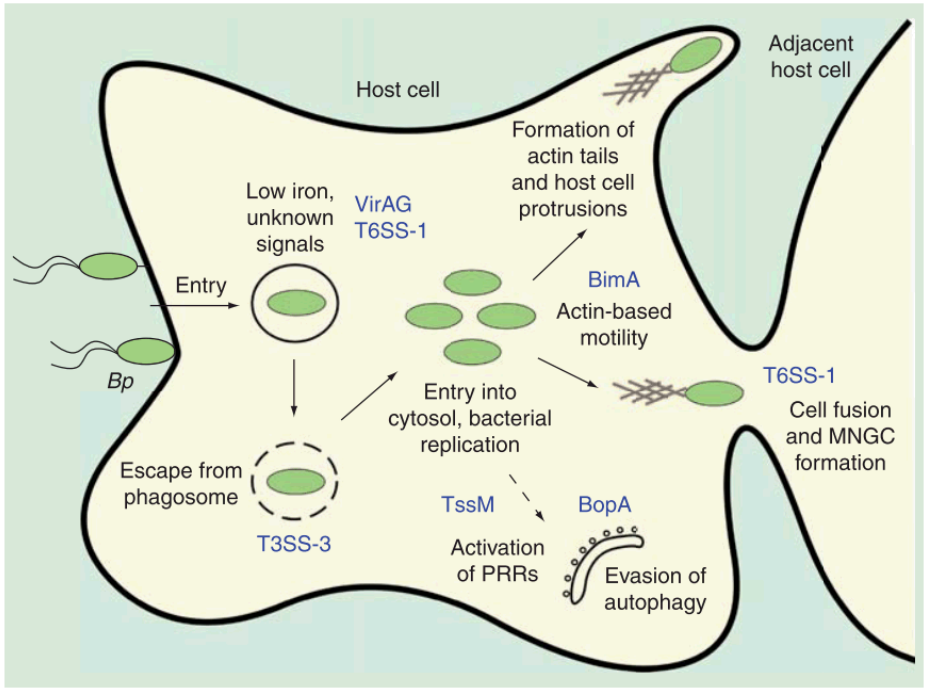
The life cycle of *Burkholderia pseudomallei*

- Life cycle involves:
  - Adherence and entry into host cells
  - Phagosome escape
  - Cytosolic replication
  - Actin propelling within the cell
  - Spreading to neighboring cells
  - Lysis of fused cells and release of bacteria



How to inhibit *Burkholderia pseudomallei*

1. Directly target the bacteria
2. Enhance the host response to the bacteria

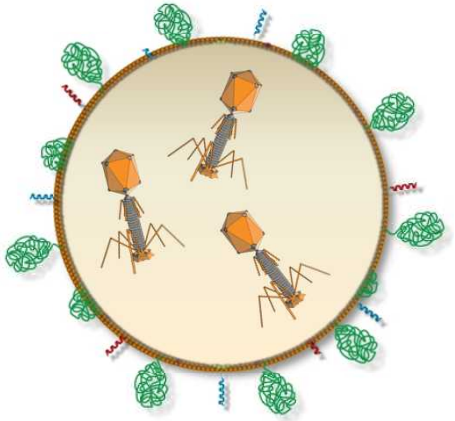


1. Targeting *Burkholderia* using CRISPR

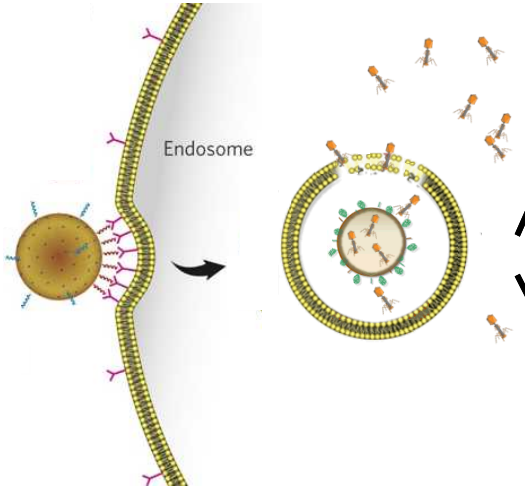
- Delivery of CRISPR antimicrobials requires two steps:
  - Delivery of CRISPR components directly to the bacteria (use a bacteriophage)
  - Delivery of bacteriophage to infected cells (encapsulate bacteriophage into a protocell)

1. Genetically Encode Bacteriophage with CRISPR/Cas9

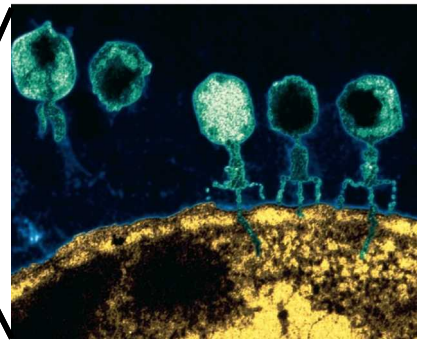
2. Encapsulate Bacteriophage into Protocells



3. Deliver Bacteriophage to Bacterially-Infected Cells



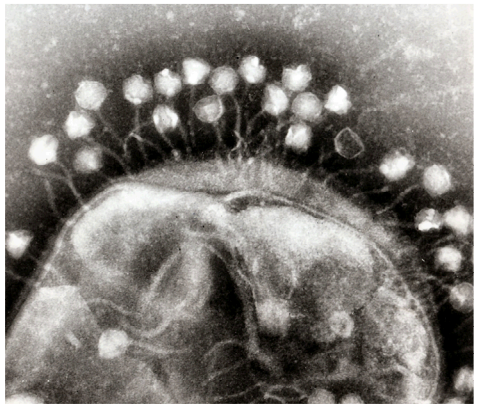
4. Bacteriophage Deliver CRISPR/Cas9 to Intracellular Bacteria

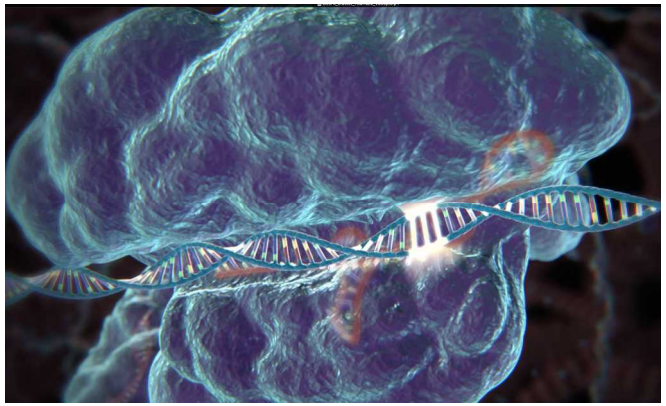


Developing *Burkholderia* antimicrobials

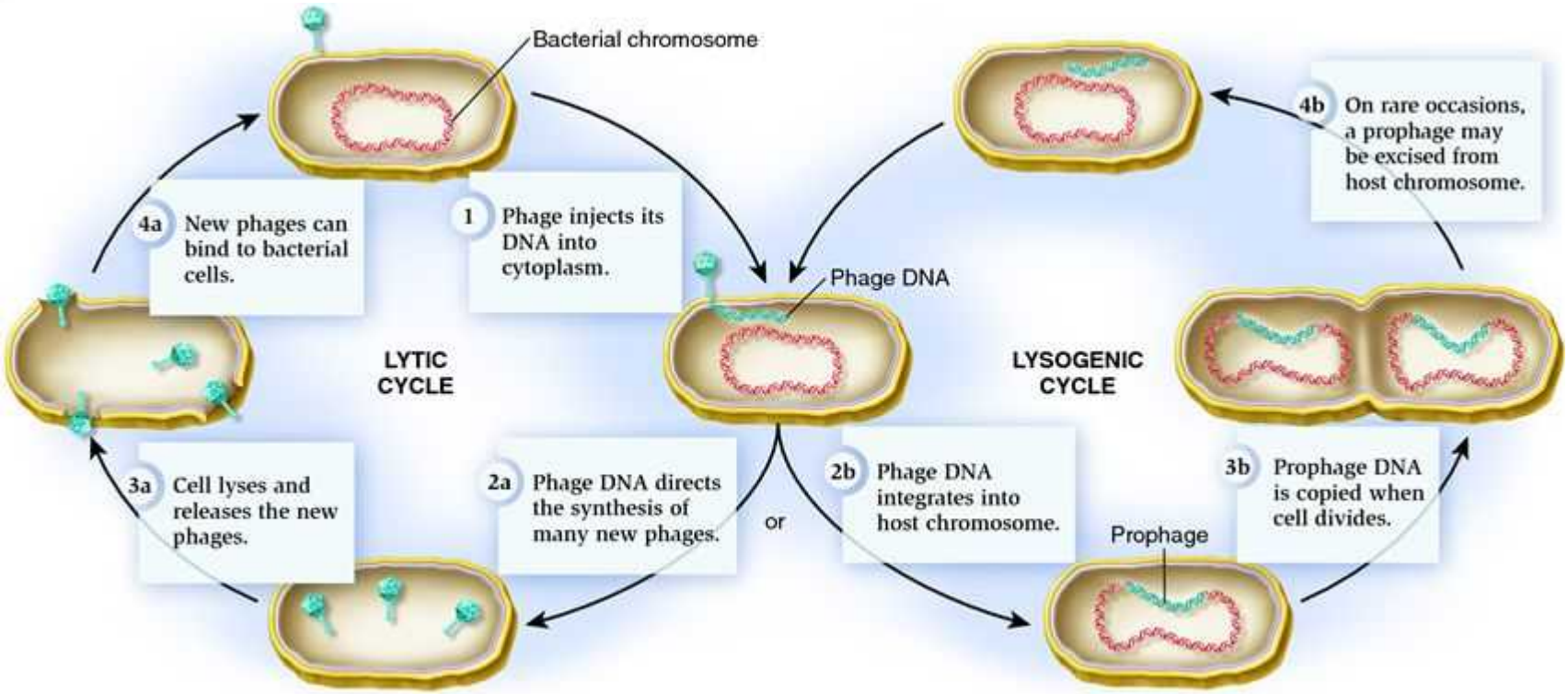
1. Isolate bacteriophage that target *Burkholderia* species
   - Screen environmental bacteriophage libraries for both lytic and lysogenic phage

2. Use CRISPR/Cas as an antimicrobial to target intracellular bacteria using protocell and bacteriophage delivery





Bacteriophage life cycle

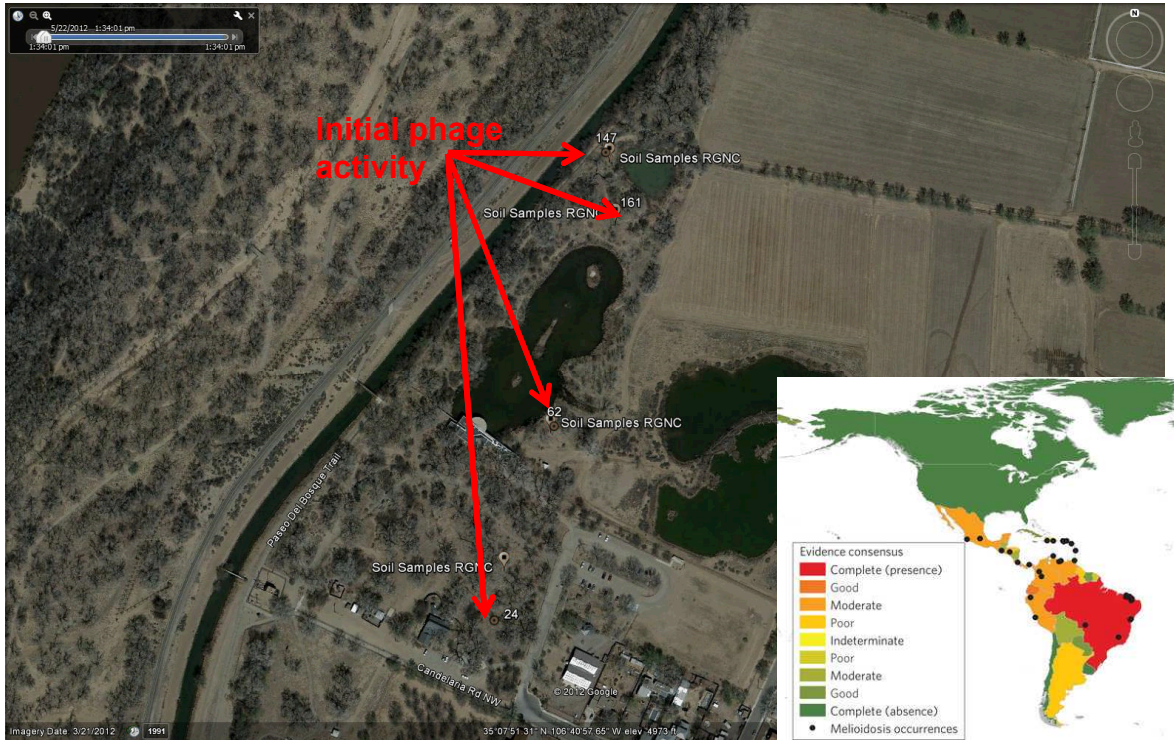


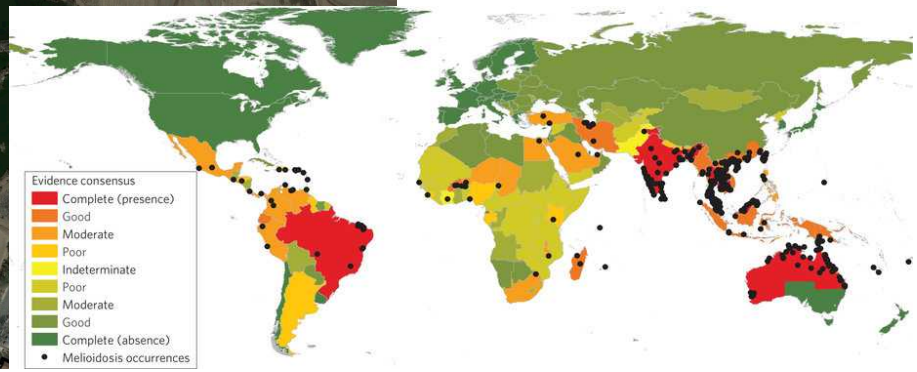
Isolation of *Burkholderia* bacteriophage

- Identify regions of interest to isolate phage based on epidemiology (Thailand and S.E. US)

Sample types:

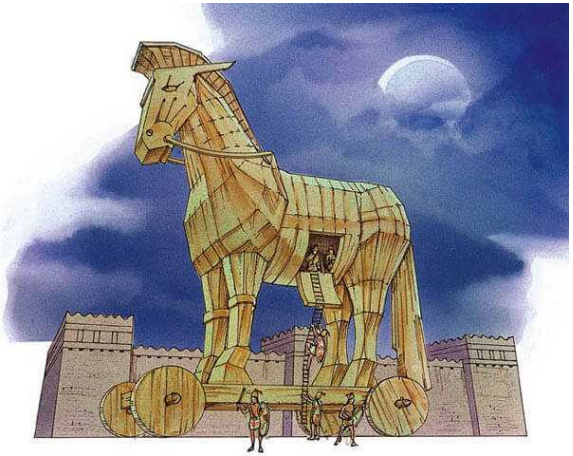
- Water
- Soil
- Sediment





Isolation of *Burkholderia* bacteriophage

- Create phage-based cocktails
  - Isolate environmental lytic bacteriophage
    - Being with RG-2 organisms (*B. thailandensis*)
      - Allows for therapeutic production
      - Cheaper
    - Expand to RG-3 pathogens (*B. pseudomallei*)
      - Technique developed at Sandia (patent disclosure)
      - Can improve lytic activity



  - Express lysogenic phage from pathogen or closely related bacteria
  - Engineer phage with CRISPR
    - These phage will carry CRISPR as a “Trojan horse” for resistant pathogens

Create a cocktail of lytic and modified lysogenic bacteriophage

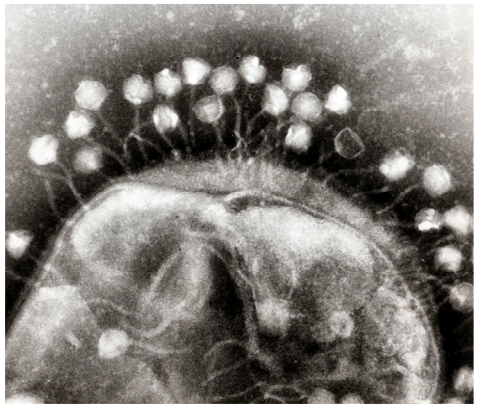
Isolation of *Burkholderia* bacteriophage

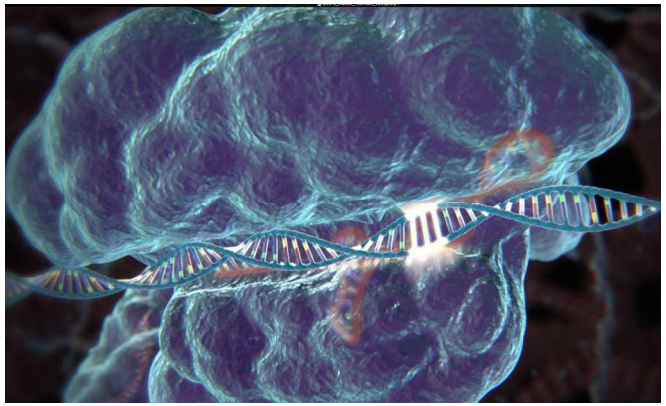
- Isolated 4 novel bacteriophage against *Bacillus anthracis*
  - Purified high titer stocks
  - Testing encapsulation and preliminary therapeutic efficacy
  - Phages are sequenced; analysis is in progress
- Isolated one potential new bacteriophage against *B. thailandensis*
  - Isolated from samples collected in Louisiana in 2015
  - Initial isolation completed
  - Testing for stability in lab and performing plaque purity
- Identified an RG-2 strain of *B. pseudomallei*
  - May use for future screening
  - May also be used for host range expansion experiments

Strategy for Developing *Burkholderia* Antimicrobials

1. Isolate bacteriophage that target *Burkholderia* species
   - Screen environmental bacteriophage libraries for both lytic and lysogenic phage

2. Use CRISPR/Cas as an antimicrobial to target intracellular bacteria using protocell and bacteriophage delivery





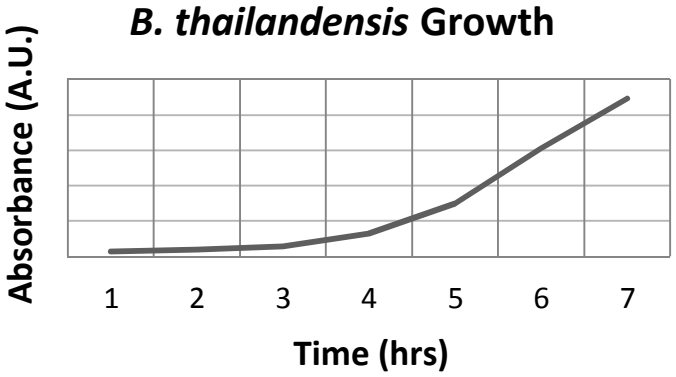
Strategy for Using CRISPR as an Antimicrobial

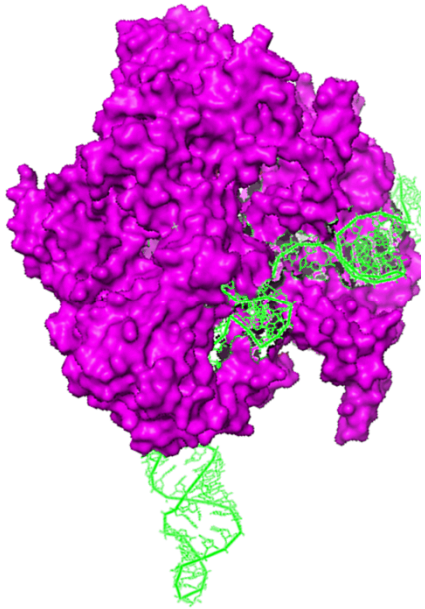
- Overall Goal: Use CRISPR/Cas as an antimicrobial to target intracellular bacteria using protocell and bacteriophage delivery
  - *Disrupt gene expression permanently*
- Use a less-virulent homolog of *B. pseudomallei*
- Begin by targeting a few essential genes in *Burkholderia thailandensis/pseudomallei* (no off targets)
  - FabH (fatty acid and phospholipid metabolism)
  - Isochorismate (iron uptake)
  - KDOP Synthase (synthesis/degradation of lipo- and polysaccharides)
- Build a bacterial targeting library (~100 genes)

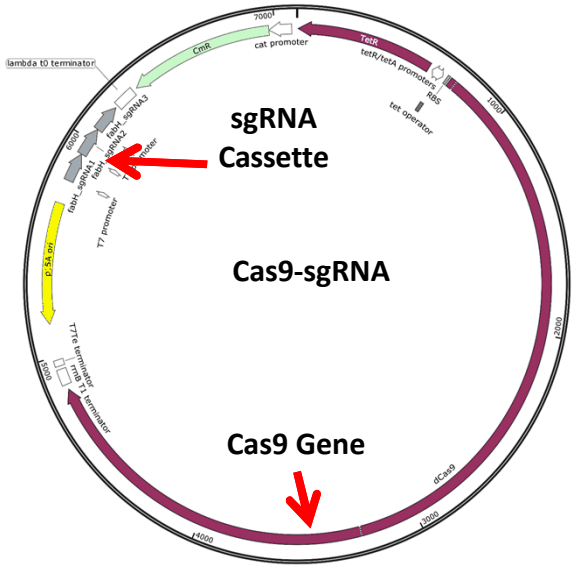
L. Baugh, *et al.*, PLoS One, 2013, 8(1): e53851

Delivering CRISPR/Cas9 to *B. thailandensis*

- Test efficacy of CRISPR targets by directly delivering CRISPR components
  - Deliver ribonucleoprotein complexes (working out protocols)
  - Deliver plasmid encoding CRISPR and Cas9



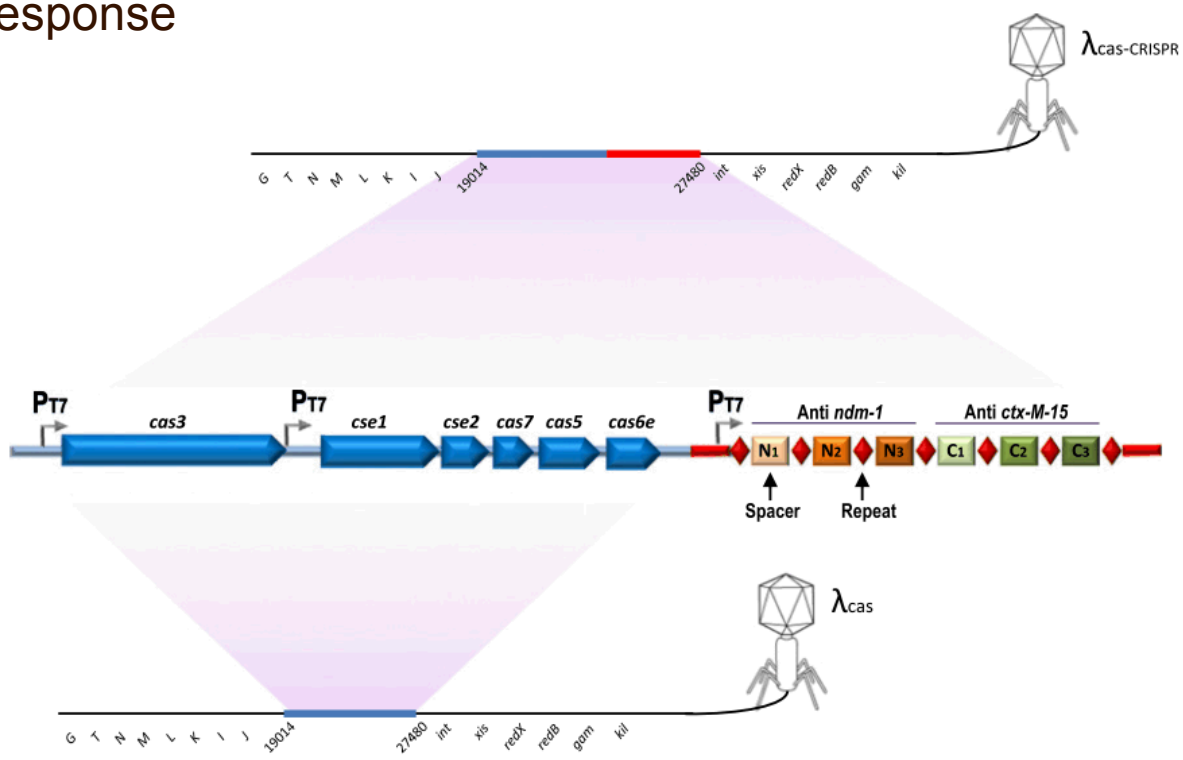




PDB Structure: L. Baugh, *et al.*, PLoS One, 2013, 8(1): e53851

Putting it All Together: Delivering CRISPR/Cas9 with Phage

- Once a phage is identified and targets confirmed:
  - Incorporate CRISPR/Cas system into bacteriophage genomes
  - Design a library of gene targets to determine most effective inhibitory response



I. Yosef, *et al.*, PNAS, 2015, 112(23): 7267-72.

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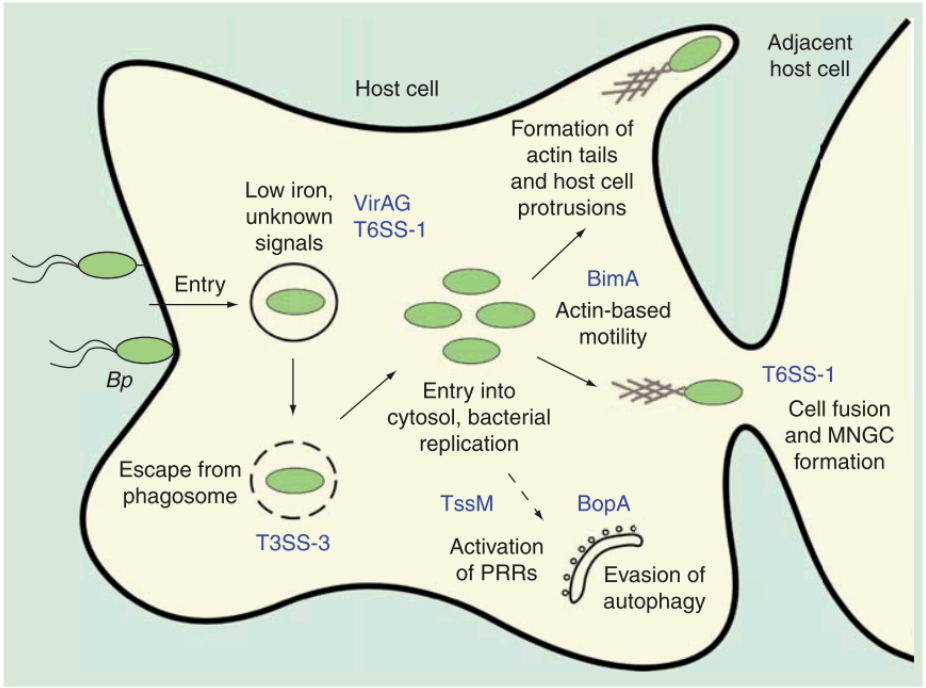
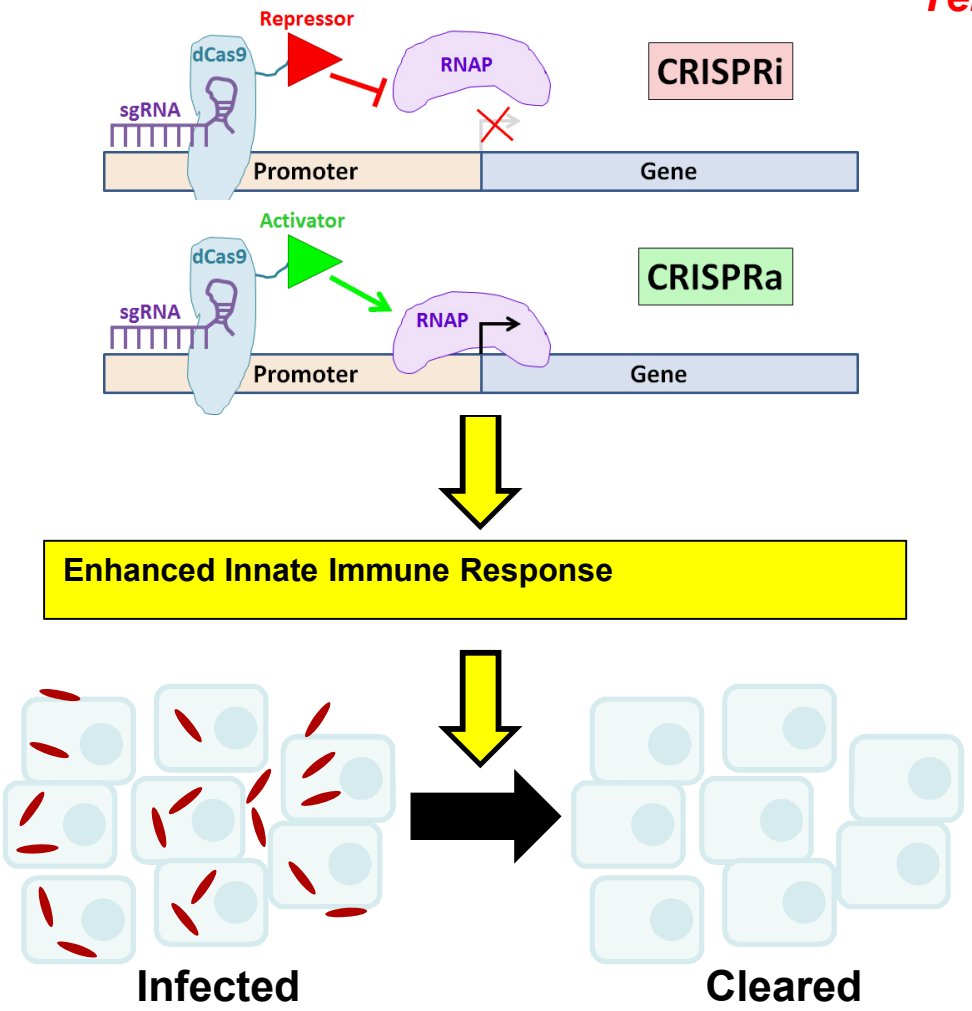


J. Stone, *et al.*, Expert Review of Anti-infective Therapy, 2014, 12(12): 1487-99

CRISPRi/a Host-directed Therapy for Infections



Temporarily disrupt gene expression

- CRISPRa/i: uses an inactive Cas9 that will bind to the target genomic loci, but will not cleave
  - Fused to an activator or repressor protein to alter gene expression
- CRISPRi: temporarily inactivates expression of gene of interest
- CRISPRa: temporarily activates expression of gene of interest

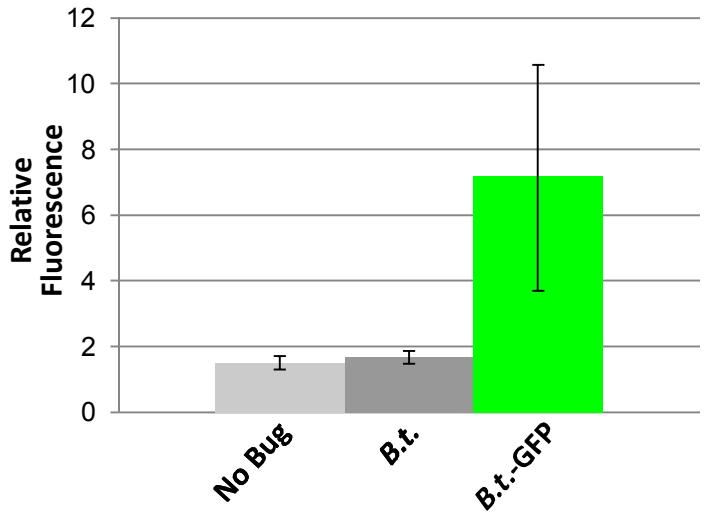
Searches for defense-enhancing CRISPRi/a constructs

1. Reverse genetics:
   - Target genes likely to mediate defense against *Burkholderia*
   - Screen for immunostimulants that enhance defense and then target genes mediating the effect

2. Forward genetics:
   - Screen knockout mutants for enhanced defense and then target the affected genes with CRISPRi to phenocopy the effect
   - Screen CRISPRa library for constructs that enhance defense

Developing *in vitro* models of *Burkholderia* infection

- Host cells: Human airway epithelial cells and monocyte-derived macrophages
  - Initial screens carried out using cell lines: A549 and THP-1
  - Further test active constructs using primary cells
- Pathogens: *B. thailandensis* (BSL-2) & *B. pseudomallei* (BSL-3)
  - Strains constitutively express GFP (MicrobiologyOpen 3:610 '14)
- Infection Model #1: Measure Burkholderia internalization/proliferation
- Infection Model #2: Measure host cell viability/proliferation



Infection: A549 +/- bug
- **MOI 500**
- **1 h internalization**
- **Antibiotics added to medium**
- **96 h intracellular replication**

Detection: 485 nm Ex → 538 nm Em
- **16 independent infection assays.**
- **Column: Mean fluorescence.**
- **Error bar: Range of fluorescence values.**

Target genes known to mediate defense against *Burkholderia*

1. Identify pathways that contribute to host defense against bacterial infection
    - Include pathways:
        - Normally elicited during infection
        - Previously manipulated in order to successfully combat infection
    - Prioritize pathways associated with defense against Burkholderia > Gm^- > other bacteria
2. Within each pathway of interest, identify genes that:
    - Primarily/only impact innate immunity (knockout/knockdown/overexpression phenotype)
    - Show basal expression levels conducive to desired manipulation:
        - High basal expression can be knocked down by CRISPRi
        - Low basal expression can be boosted by CRISPRa

Pathway	CRISPRa Targets	CRISPRi Targets
Receptor-Mediated Pathogen Recognition	STING, NIK, TAK1	SARM, MKP-1, IRAK-M, A20, TRIAD3A, RP105, LILRA1-6, SIGIRR, TRAILR, ST2L, SRA, SHP-1, SHP-2, SHIP, SOCS1, PI3K, CYLD, TOLLIP, PPARG, VEGFR-3, VEGF-C, AKT1, NLRC5, MCPIP1, LGR4, TRIM30a, LRRC33, LRRFIP2
IFNγ-Mediated Activation & Recruitment	miR-122, miR-185 miR-221	NOD2, SOCS3, CIS
Reactive Oxygen Species Production	CYBA, CYBB, NOXO1, NOXA1, NCF1, NCF2	HDAC6, NRROS
Phagosome/Lysosome Biogenesis	AGS3, SYK, CTSL, CTSS	-
Nitric Oxide Production	iNOS, Ass1, Asl, NKLAM	SIRPa
Autophagy	CST9, MRP8, MRP14, miR-7, miR-155	MCL-1, MTORC1, RHEB, RICTOR, RPS6KB2
Inflammatory Prostaglandin Production	-	COX2

Identify protective immunostimulants and then target pathways/genes likely to mediate the protective effect

Receptor Class	Primary	Secondary	Agonist	Agonist Description	Agonist Origin
TLR2	TLR1/2 TLR2/6		PGN-BS	Peptidoglycan	*Bacillus subtilis*
			LTA-BS	Lipoteichoic acid	
			LM-MS	Lipomannan	*Mycobacterium smegmatis*
		Dectin-1	Zymosan	Cell Wall Extract	*Saccharomyces cerevisiae*
	TLR1/2		Pam3CSK4	Triacylated Lipoprotein	Synthetic
	TLR2/6		Pam2CSK4	Diacylated Lipoprotein	
			FSL-1		
TLR3	TLR3		Poly(I:C) LMW	Low Molecular Weight dsRNA	Synthetic
			Poly(I:C) HMW	High Molecular Weight dsRNA	
			Poly(A:U)	Poly(A:U) dsRNA	
TLR4	TLR4	TLR2	LPS-B5	Lipopolysaccharide	*Escherichia coli* O55:B5
			LPS-RS		*Rhodobacter sphaeroides*
			LPS-SM		*Salmonella minnesota* R595
			MPLA-SM	Monophosphoryl Lipid A	
TLR5	TLR5		FLA-BS	Flagellin	*Bacillus subtilis*
TLR7, TLR8	TLR7		CL264	Adenine Analog	Synthetic
			Imiquimod	Imidazoquinoline Compound	
		TLR8	Gardiquimod	Imidazoquinoline Compound	
			R848	Imidazoquinoline Compound	
	TLR8		ssRNA40	GU-rich ssRNA	
			ssPolyU	Poly-U ssRNA	
			ORN 06	GU-rich ssRNA	
TLR9	TLR9		Ec-ssDNA	CpG ssDNA	*Escherichia coli* K-12
			ODN 2216	Class A CpG dsDNA	Synthetic
			ODN 2006	Class B CpG dsDNA	
			ODN 2395	Class C CpG dsDNA	
Dectin-1	Dectin-1		Curdlan AL	Beta-1,3-glucan	*Alcaligenes faecalis*
			BGP	Beta-glucan peptide	*Trametes versicolor*
			WGP	1,3/1,6 beta-glucan	*Saccharomyces cerevisiae*
			Furfurman	Cell Wall Extract	*Malassezia furfur*
NOD1, NOD2	NOD1, NOD2		PGN-ECndi	Insoluble Peptidoglycan	*Escherichia coli* K-12
			PGN-ECndss	Soluble Peptidoglycan	
	NOD1		C12-iE-DAP	Acylated Peptidoglycan Dipeptide	Synthetic
		NOD2	M-TriDap	Peptidoglycan Degradation Product	
	NOD2		M-TriLys	Muramyl Tripeptide	
			L18-MDP	Muramyl Dipeptide Derivative	
NLRP3	NLRP3		Chitosan	Deacylated Derivative of Chitin	*Agaricus bisporus*
			MSU	Monosodium Urate (Uric Acid) Crystals	*Homo sapiens*
AIM2	AIM2	RIG-I	Poly(dA:dT)	Double-Stranded B-DNA	Synthetic
			Poly(dG:dC)	Double-Stranded Z-DNA	
RIG-I, MDA-5	RIG-I, MDA-5		Poly(I:C) LMW	Low Molecular Weight dsRNA	Synthetic
			Poly(I:C) HMW	High Molecular Weight dsRNA	
	RIG-I		5'ppp-dsRNA	5'-Triphosphate dsRNA	
cGAS, STING	cGAS		ISD	Bacterial dDNA	Synthetic
			VACV-70	Viral dsDNA	
		DDX41, IFI16	HSV-60	Viral dsDNA	
	STING		2'3'-cGAMP	Cyclic GMP-AMP	
			3'3'-cGAMP	Cyclic GMP-AMP	
			c-di-GMP	Cyclic di-GMP	
Mincle	Mincle		TDB-HS15	*Mycobacterium tuberculosis* Cord Factor	Synthetic

1) Identify compounds that enable host cells to inhibit entry and/or intracellular replication of *Bt*-GFP
   - Screen infection assays for reduced GFP signal
   - Microtiter plate format

2) Identify pathways elicited by active compounds.
   - RNA-Seq analysis for global view of effects
   - Flow cytometry & ELISA for protein analyses

3) Target key genes in each pathway *via* CRISPRi/a

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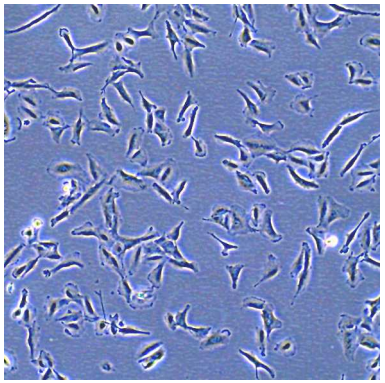
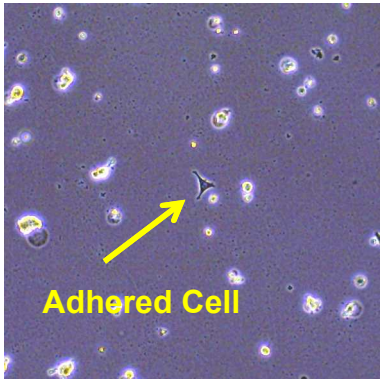
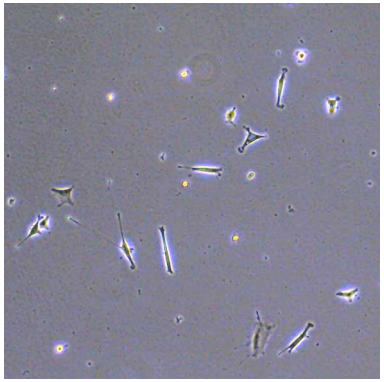


Pre-Infection



24 h Infection



48 h Recovery

Infection: A549-Cas9 +/- bug

- **MOI 4000**
- **1.5 h internalization**
- **Antibiotics added to medium**
- **24 h intracellular replication**
- **Adhered host cells recovered & cultured for 48 h**

Screen libraries for targets enhancing host response

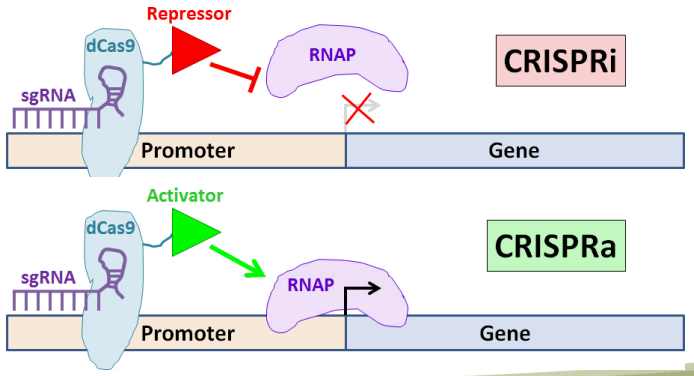
CRISPRi:

GeCKO Genome-scale CRISPR Knock-Out

1. Screen gene knockout library (GeCKO v2) for sgRNA targets that enhance host cell viability/proliferation assay
   - Infect cells, recover survivors, sequence/identify sgRNA
   - Initial screen for Bt + A549-Cas9 is in progress
2. Identify the genes targeted by protection-conferring sgRNA
3. Design CRISPRi constructs that target these genes, & identify those that similarly confer protection

CRISPRa:

1. Screen pre-existing libraries (Weissman, SAM) for protection-conferring constructs
   - Do sgRNA protospacer sequences effectively target the gene promoters in A549 & THP-1?
2. Build and screen new cell-specific CRISPRa libraries



Parallel Approaches for Designing Antimicrobials

1. Targeting bacteria directly using CRISPR
   - Identify lytic and lysogenic bacteriophage specific for *Burkholderia*
   - Deliver CRISPR/Cas9 components (DNA and RNPs) to screen for effective sgRNA targets (*in vitro)*
   - Encode CRISPR/Cas9 components into the identified lysogenic bacteriophage for targeted delivery
   - Use bioinformatics to screen bacteriophage libraries for endogenous CRISPR/Cas sequences
     - Alter the CRISPR sequences to target our genes of interest

2. Targeting the host-directed response
   - Forward Genetics:
     - Target genes known to mediate defense against *Burkholderia*
     - Identify immunostimulants that enhance defense and then target genes mediating the effect
   - Reverse Genetics:
     - Screen gene disruption mutants for enhanced defense and then phenocopy using CRISPRi
     - Screen CRISPRa constructs for defense-enhancing activity