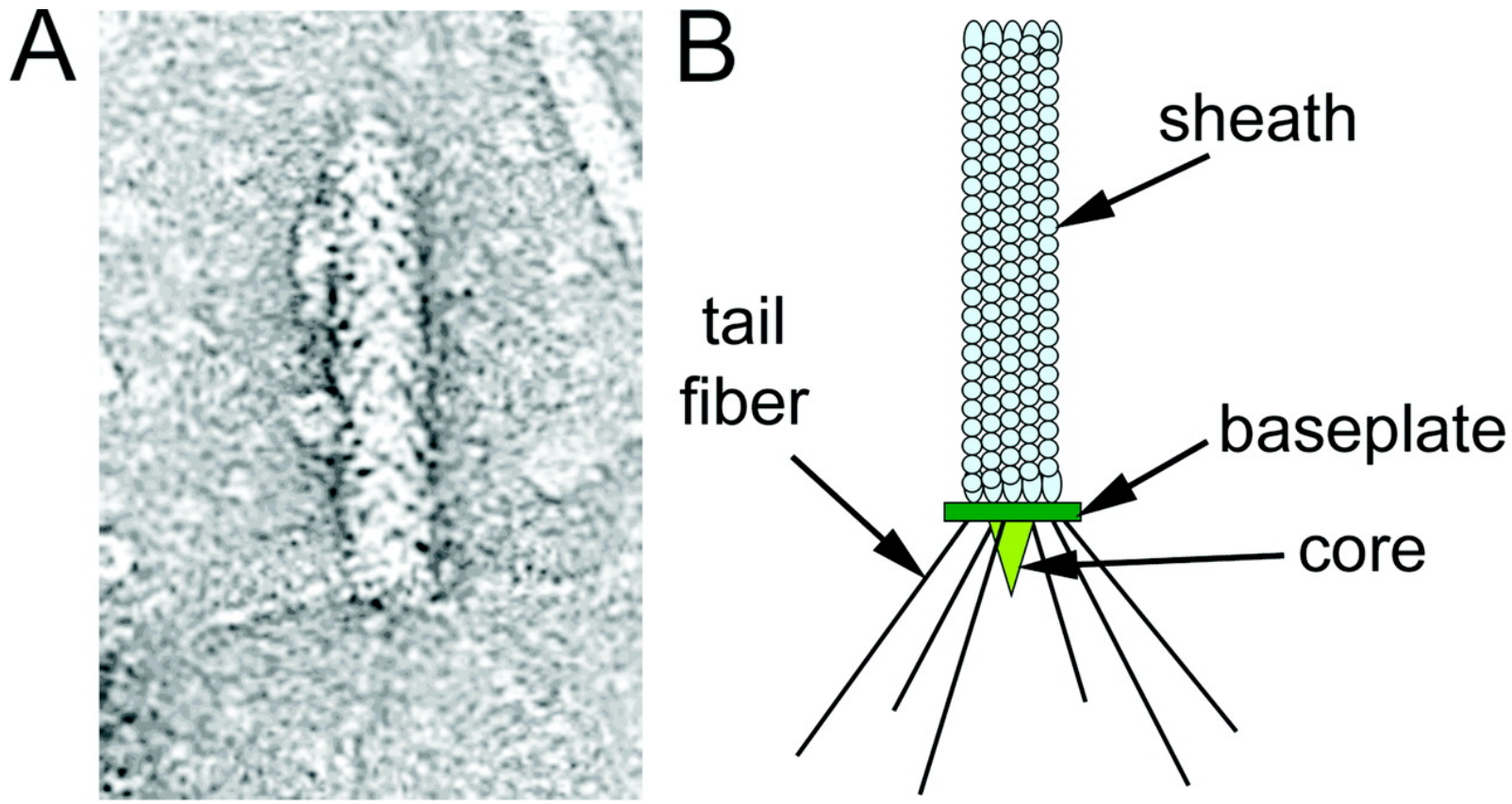
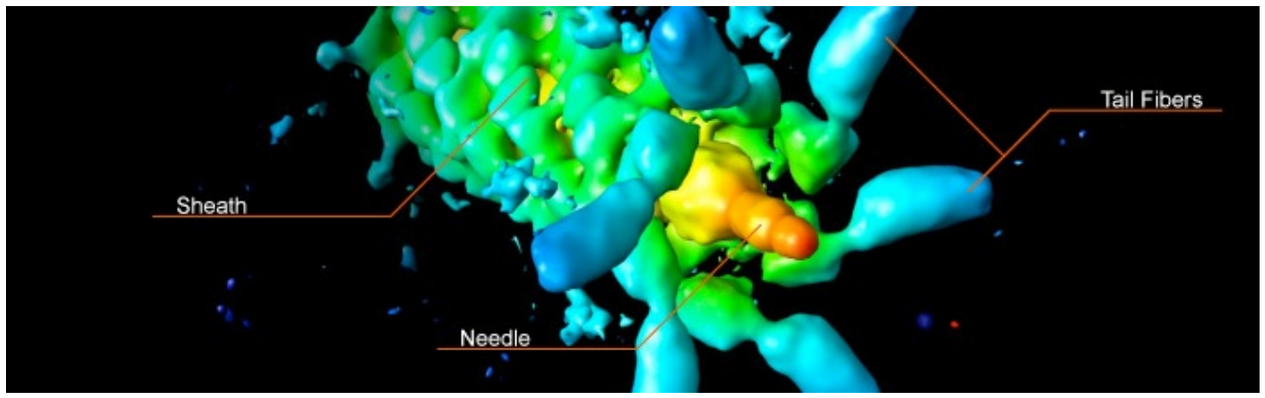
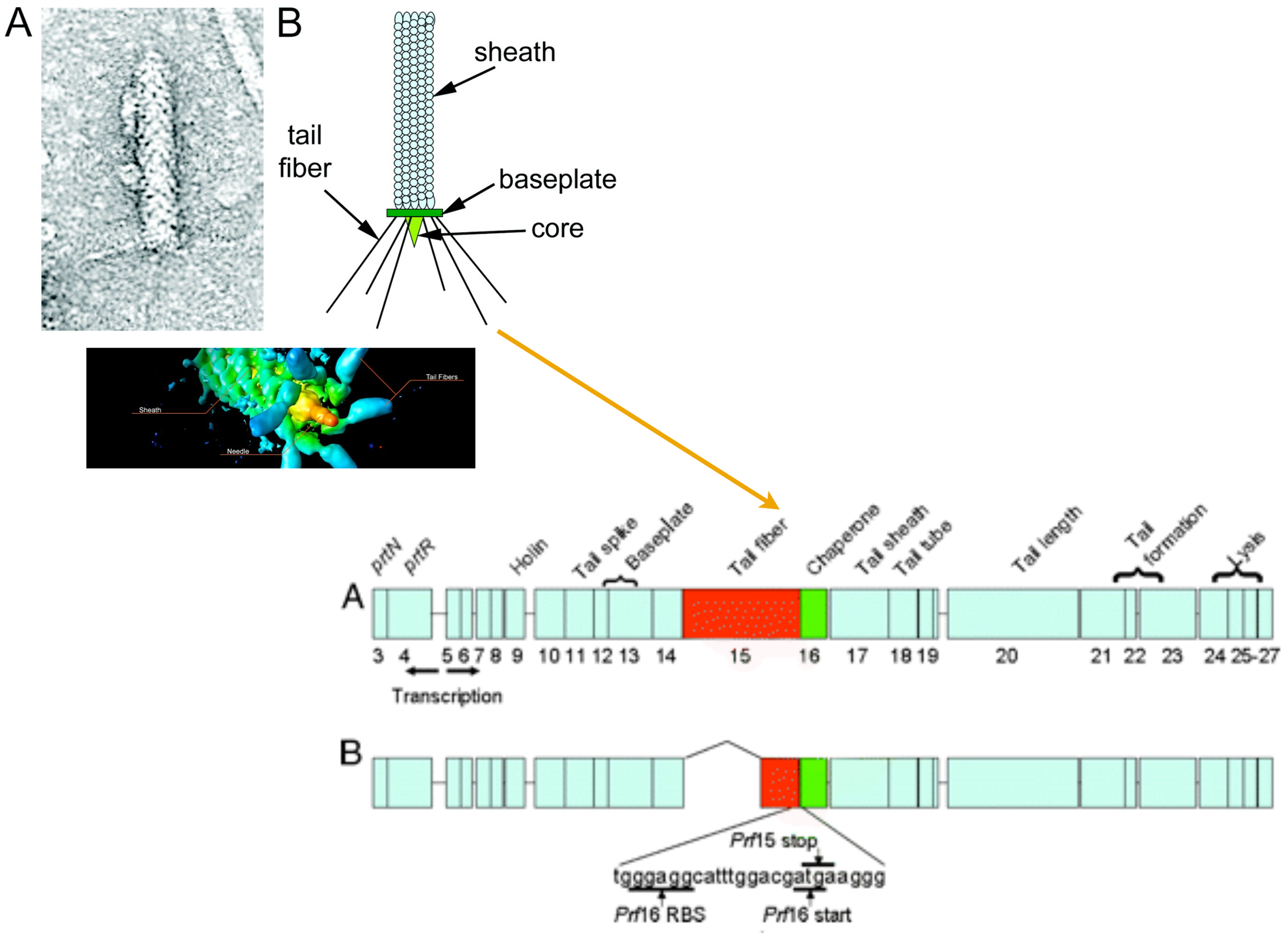
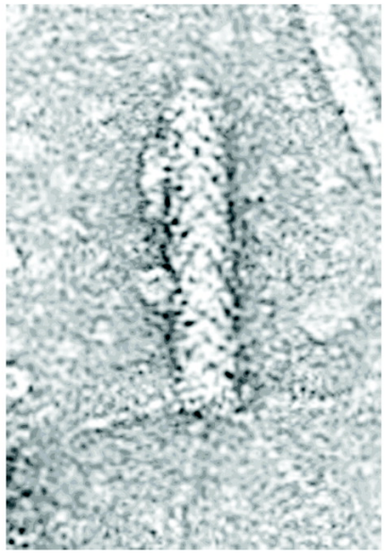




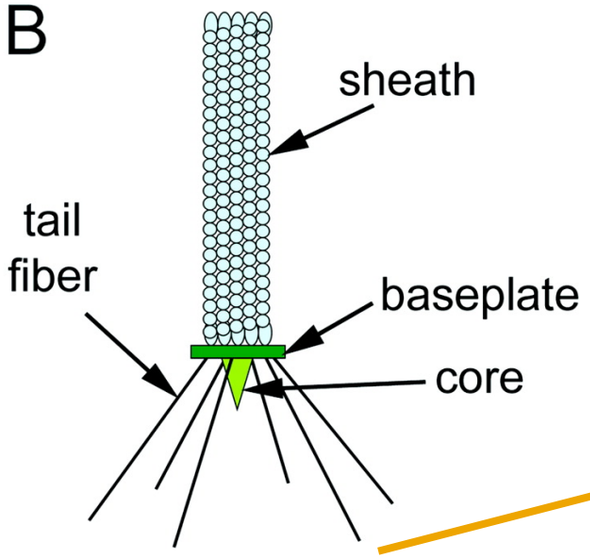
Pyocins - Phage-like proteins produced by *Pseudomonas* sp. to kill related strains

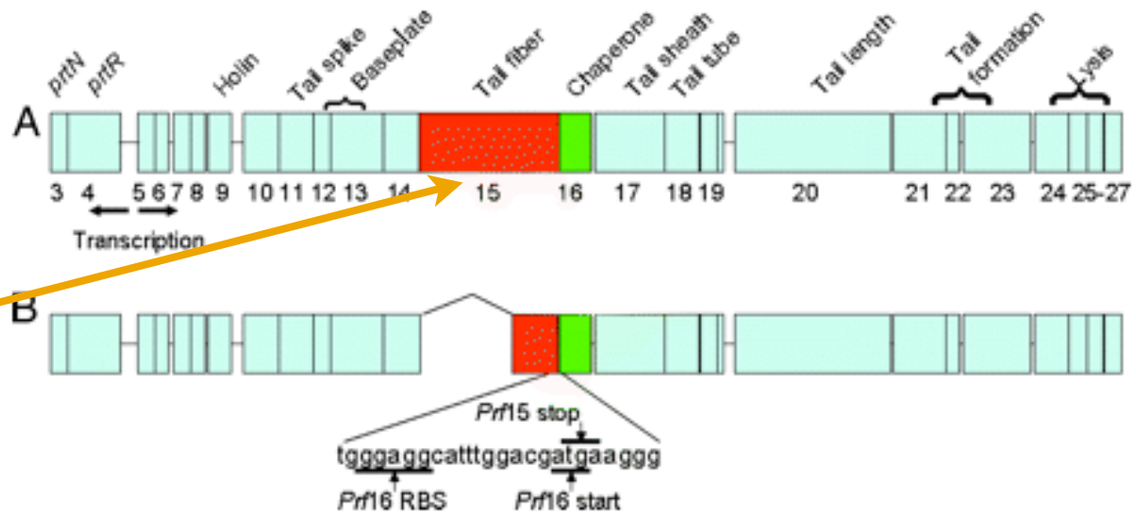

A
B
sheath
tail fiber
baseplate
core
Tail Fibers
Sheath
Needle
prtN
prtR
Holin
Tail spike
Baseplate
Tail fiber
Chaperone
Tail sheath
Tail tube
Tail length
Tail formation
Lysis
A
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25-27
Transcription
B
Prf15 stop
tgggaggcatttggacgatgaaggg
Prf16 RBS
Prf16 start

A

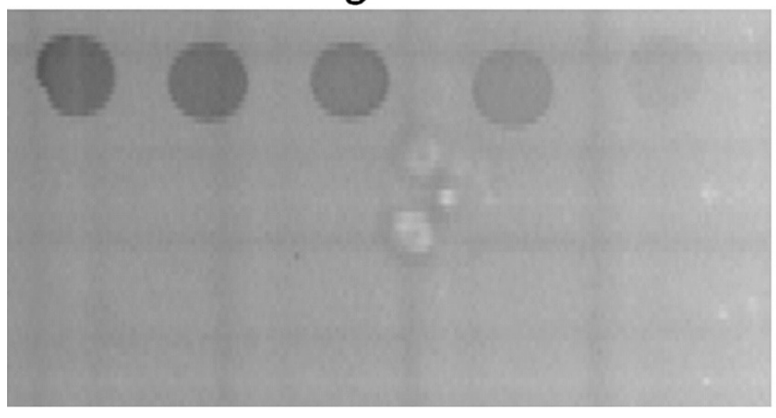


B





P. aeruginosa 13s

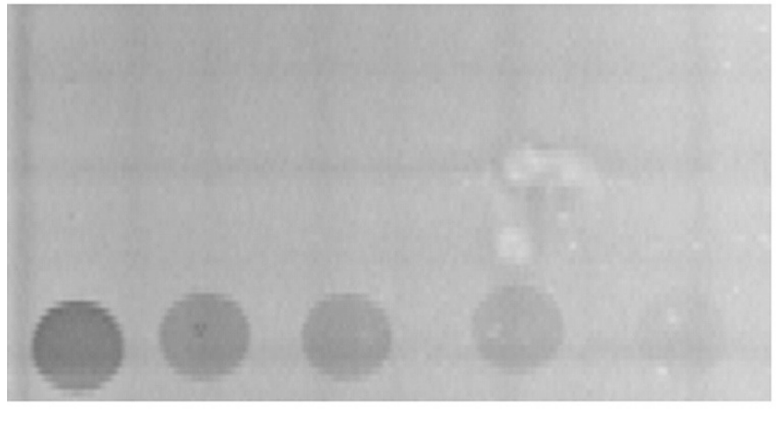


pyR2

pyR2Δprf15

pyR2-P2

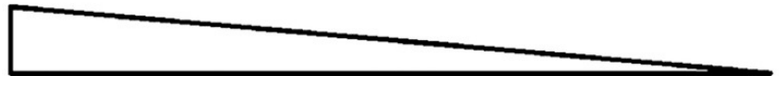
E. coli C-1a



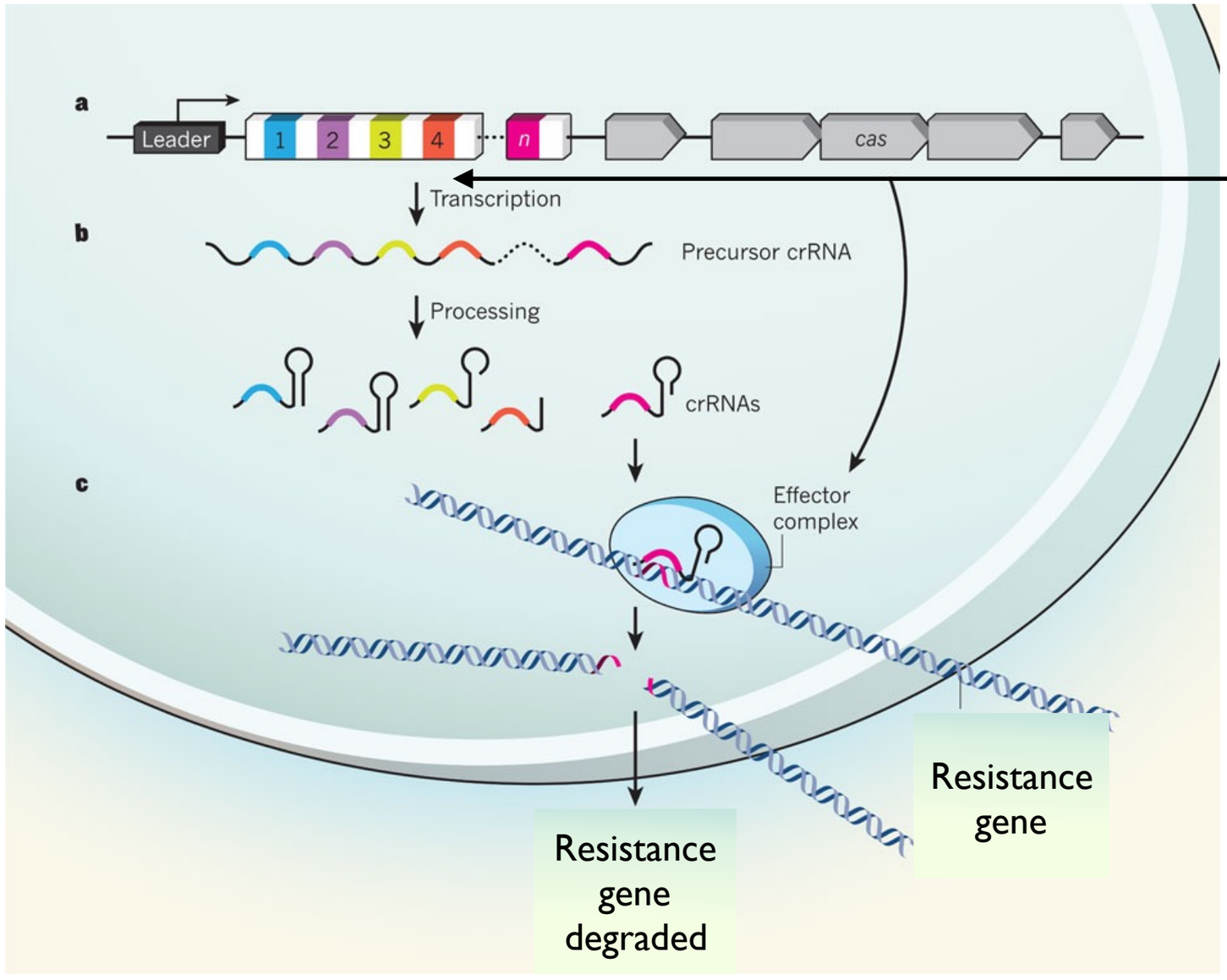
pyR2

pyR2Δprf15

pyR2-P2



[pyocin]



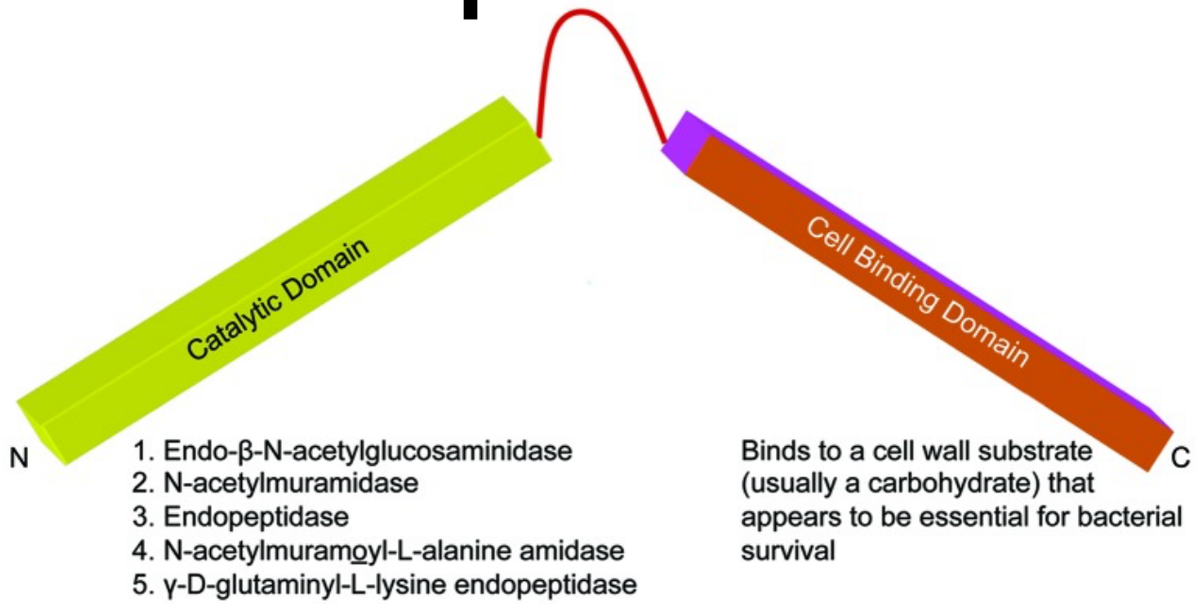
replace phage recognition sequences with resistance gene recognition ones

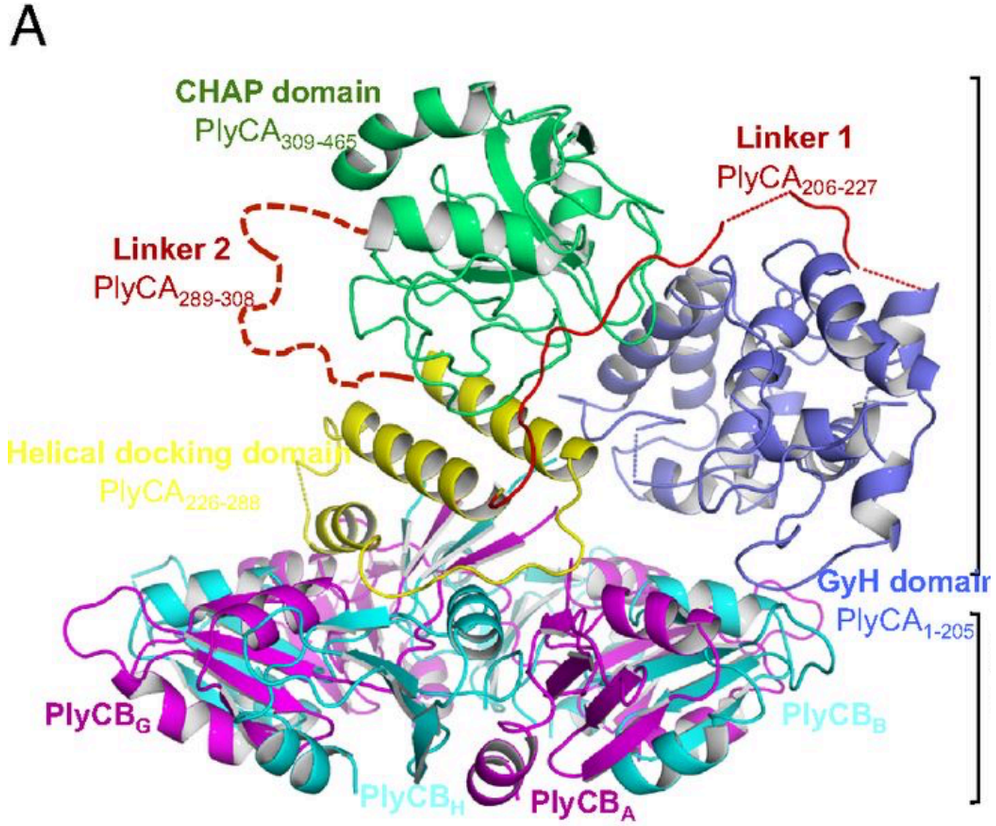
Redirecting bacterial immunity

PlyG - further development

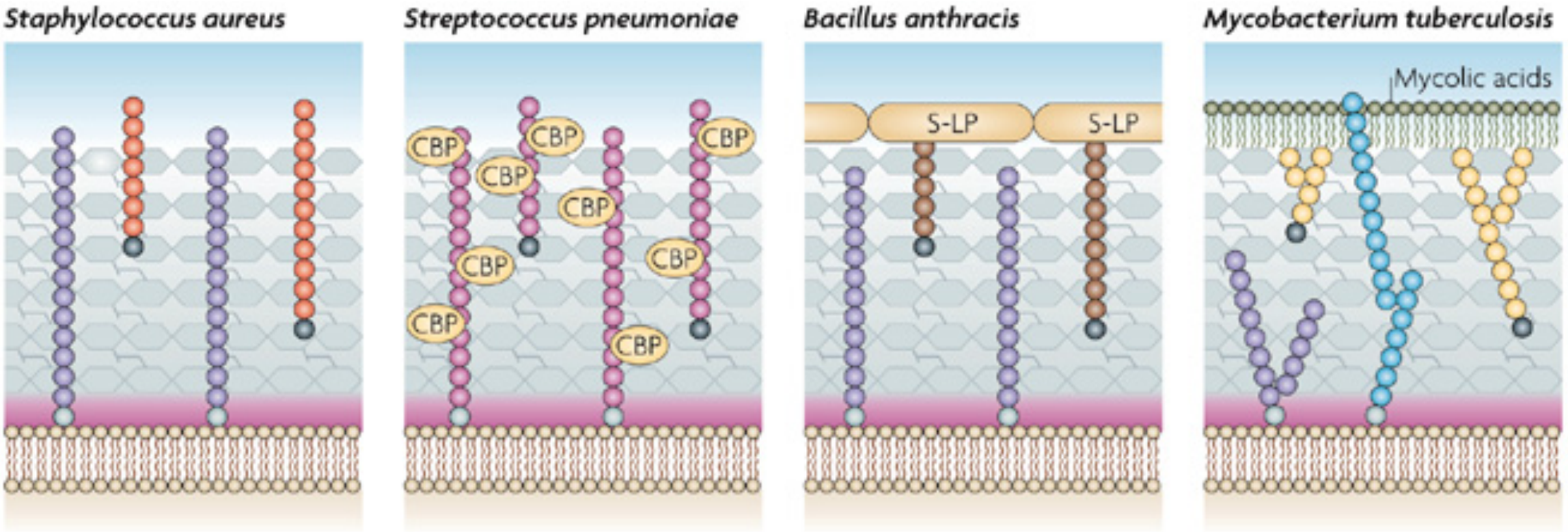
- Lysins act by disrupting binding of peptidoglycan and cell wall glycopolymers (CWGs)

- Lysins have well-conserved N-terminal peptidoglycan-cleaving domains and more divergent binding domains that recognise CWGs

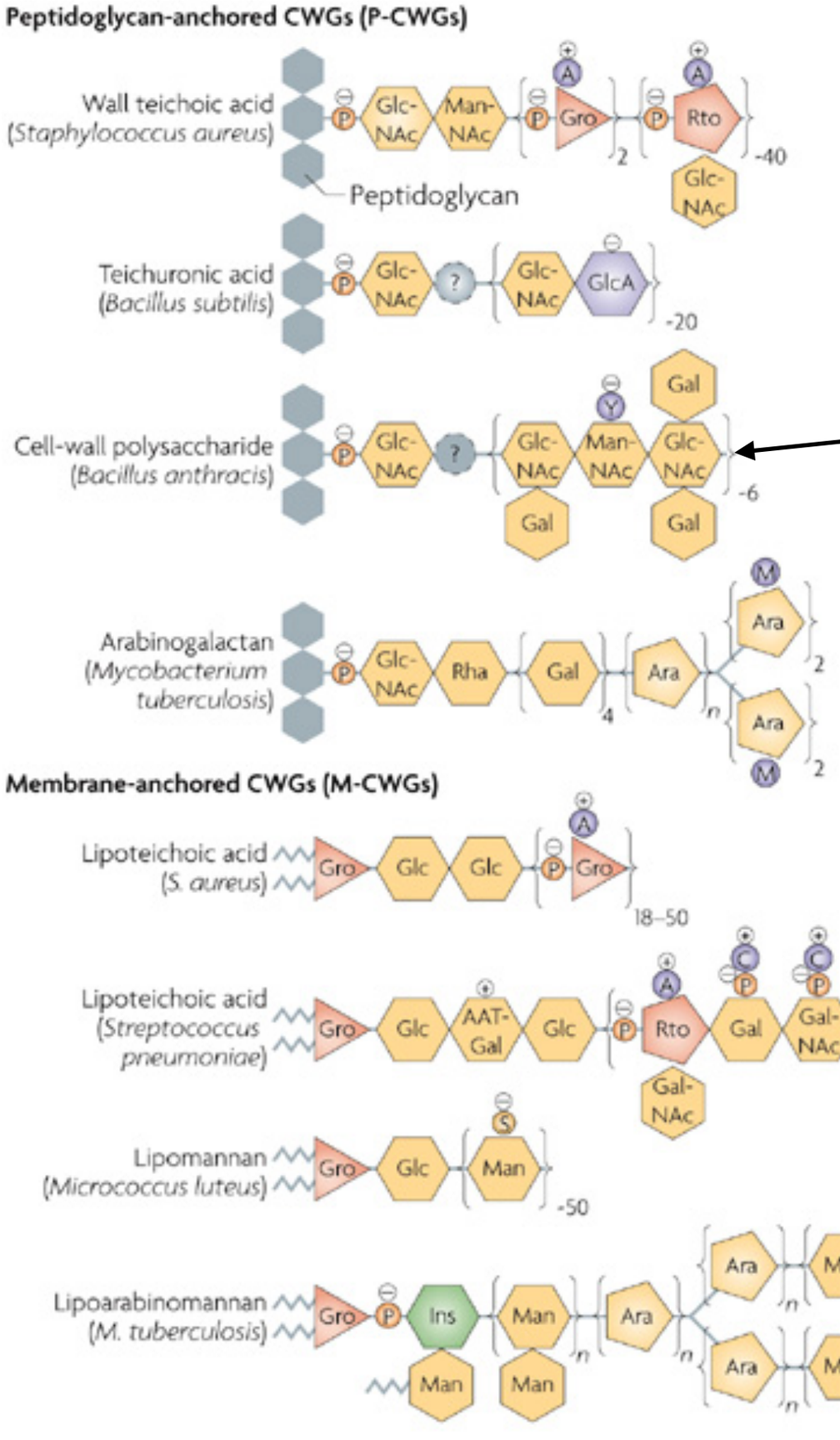




Schuch R, Pelzek AJ, Raz A, Euler CW, Ryan PA, et al. (2013) Use of a Bacteriophage Lysin to Identify a Novel Target for Antimicrobial Development. PLoS ONE 8(4): e60754. doi:10.1371/journal.pone.0060754



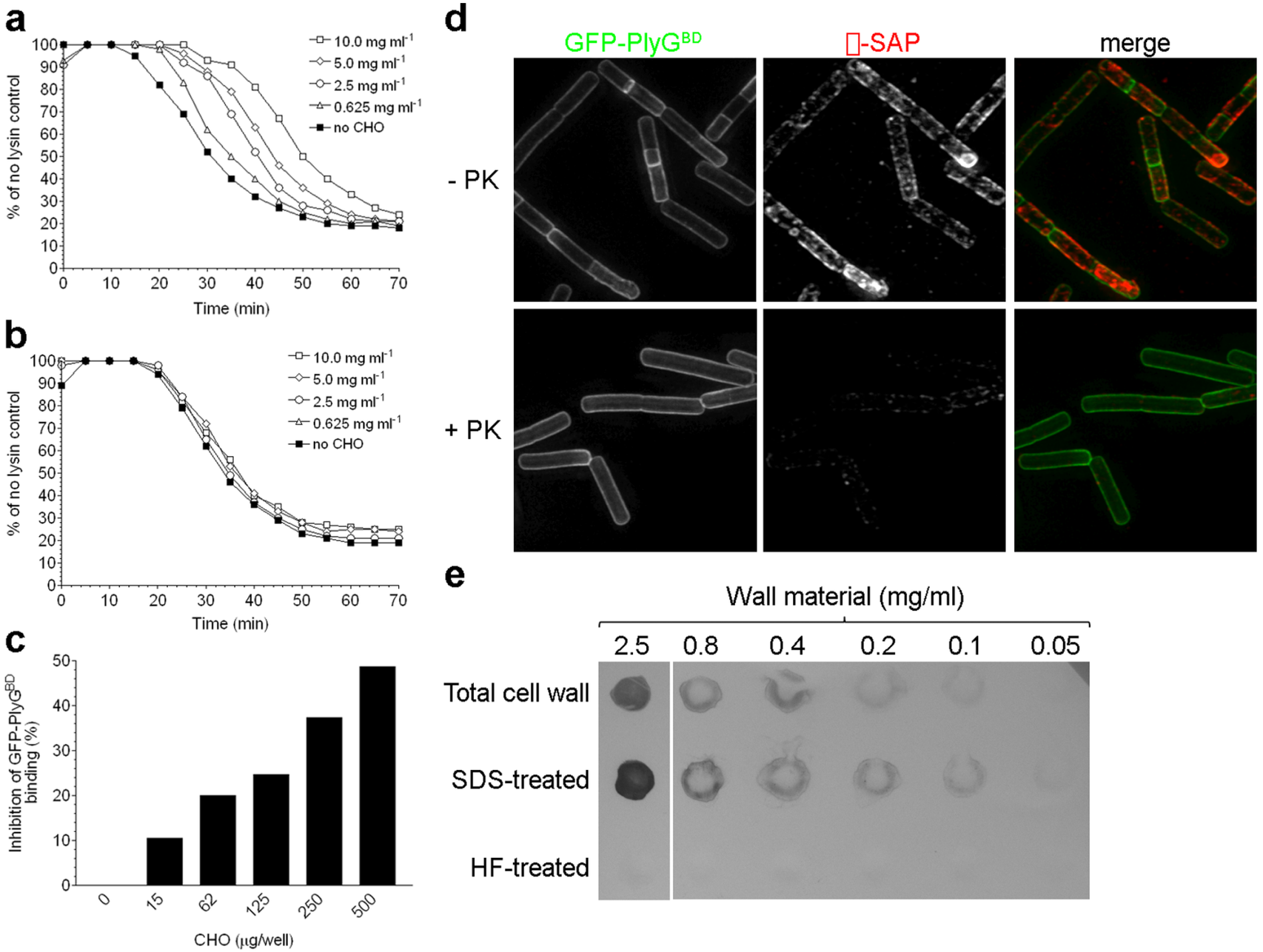
CWG polymers are shown as chains of circles within the cell wall. Differences in the composition of CWG repeating units are indicated by different colours. Linkage units that connect CWGs with peptidoglycan or lipids are shown as dark- or light-grey circles, respectively. CWGs connect choline-binding proteins (CBPs) in *S. pneumoniae*, S-layer proteins (S-LPs) in *B. anthracis* and mycolic acids in *M. tuberculosis*. Bacilli and mycobacteria often contain more than the two types of CWG shown here.



Theory

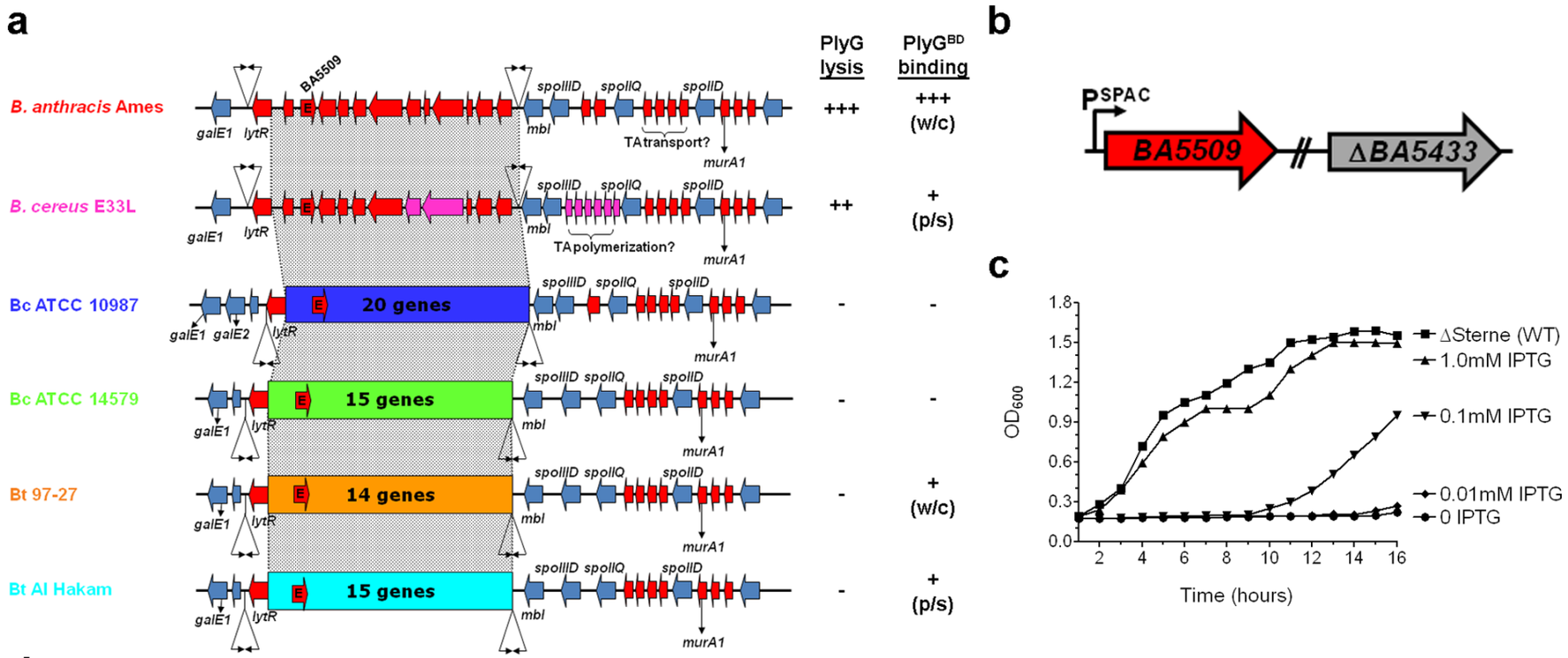
PlyG, encoded by the γ phage of *Bacillus anthracis*. PlyG cleaves *B. anthracis* peptidoglycan in a process proposed to first require binding to the CWG - In this case a Neutral Polysaccharide (NPS)

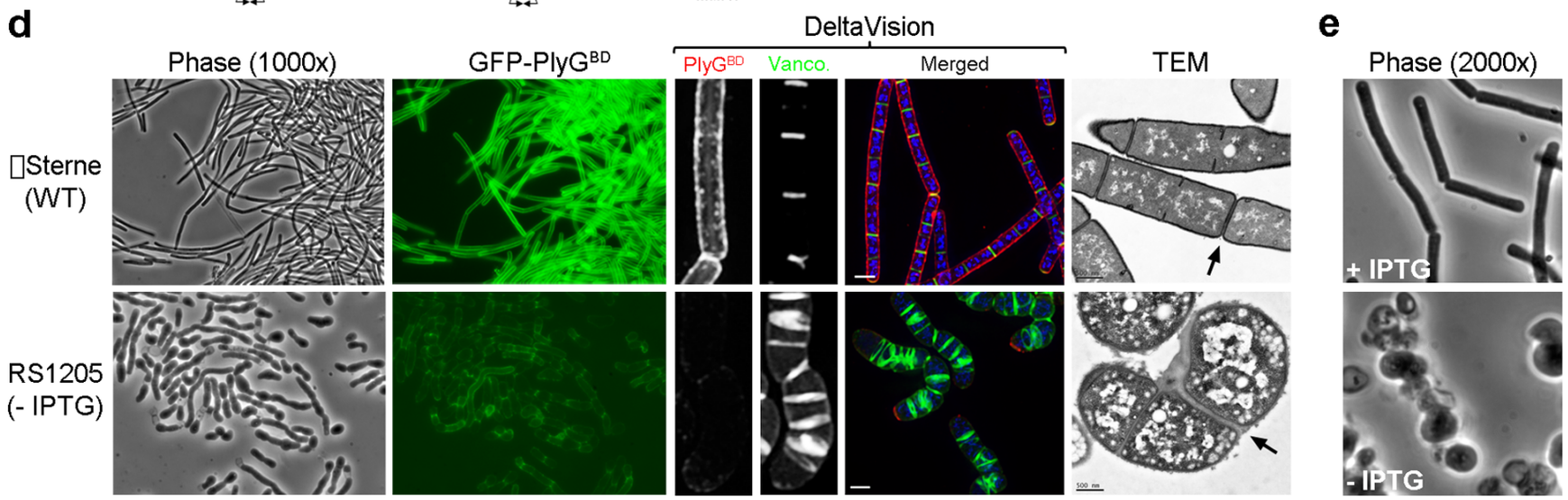
Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. Weidenmaier & Peschel Nature Reviews Microbiology 6, 276-287 (April 2008)



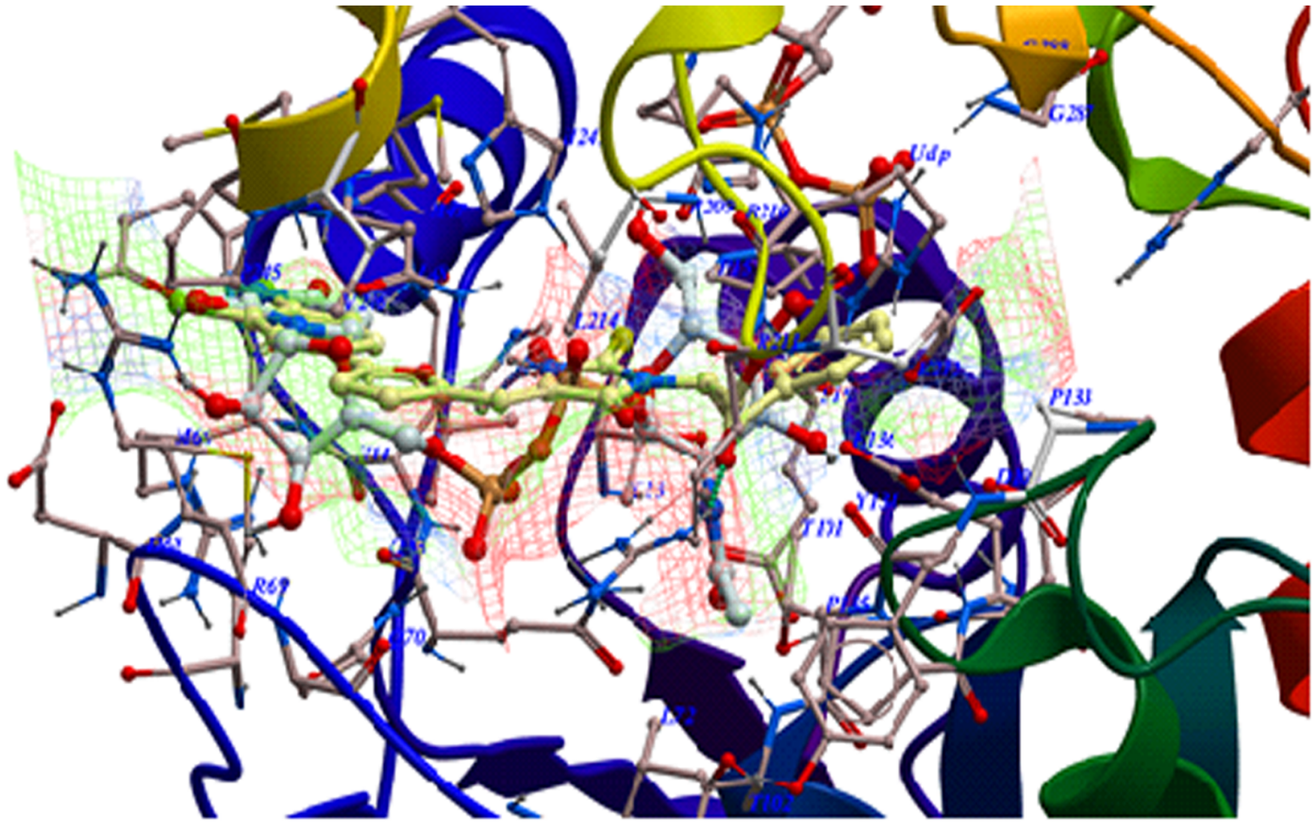
Prove PlyG binds to *B. anthracis* CWG

Figure 1. Interaction of PlyG with *B. anthracis* NPS.
(A) Dose-dependent inhibition of PlyG lytic activity after pre-incubation with *B. anthracis* CWG. (B) PlyG activity after pre-incubation with increasing amounts of the CWG from *Streptococcus pyogenes*. (C) Dose-dependent inhibition of PlyG^{BD} surface-binding after pre-incubation with *B. anthracis* CWG. (D) Deltavision images of surface-labeled *B. anthracis* with or without proteinase K treatment (+/–PK). CWG (green) was labeled with GFP-PlyG^{BD}, and the S-layer Sap protein (red) was labeled with specific antibodies and an Alexa Fluor 647-conjugated secondary antibody. (E) Dot-blot analysis of PlyG^{BD} binding to total cell wall material and both SDS-treated and Hydrofluoric acid-treated walls (removes CWG).

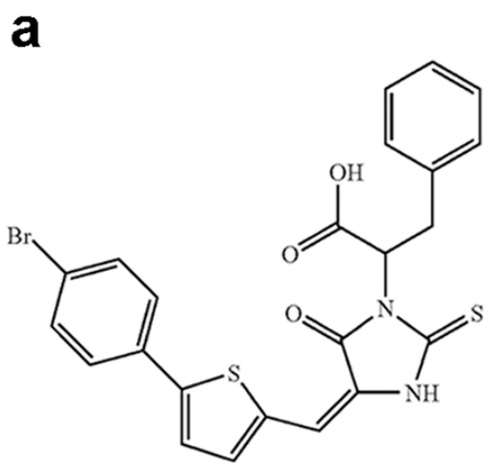


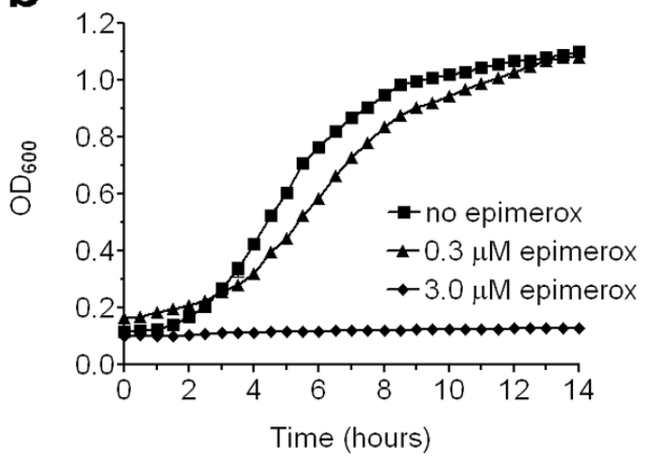


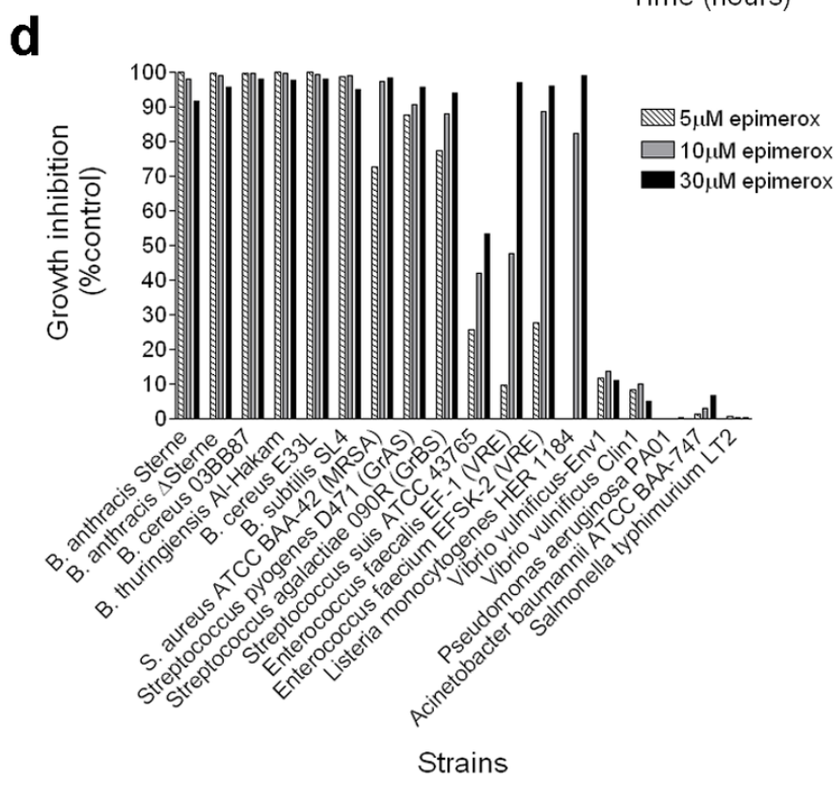
Identify genes responsible for CWGs - variable between species - identified a conserved non-hydrolyzing UDP-N-acetylglucosamine 2-epimerase (or 2-epimerase) in all - two genes present in *B. anthracis*. Place one under IPTG control (*BA5509*) and knockout the second copy (*BA5433*)

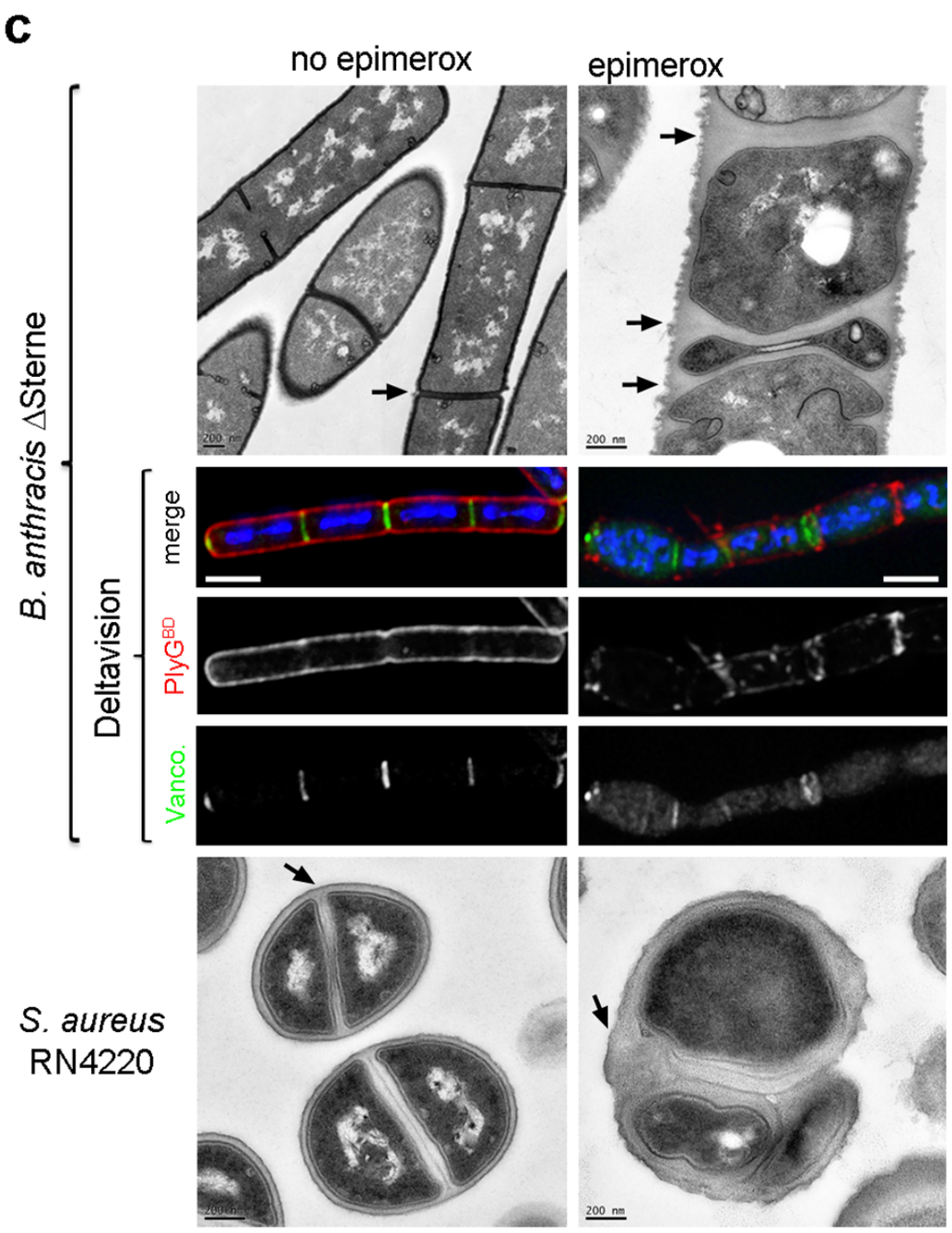


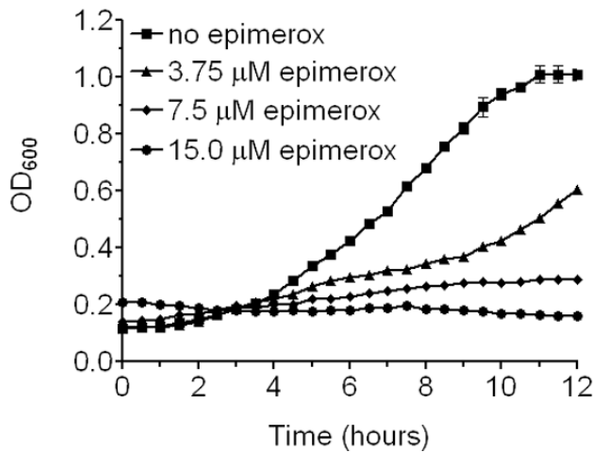
Solved the structure of 2-epimerase. Active and allosteric sites of enzymes are not present in humans. Run a virtual docking simulation of 2,000,000 small molecules. Identify and synthesise candidates with good binding energy. Run antimicrobial assays - modify structure of good candidates, rerun assays. identified a compound that was a specific inhibitor of 2-epimerase with good antibacterial activity

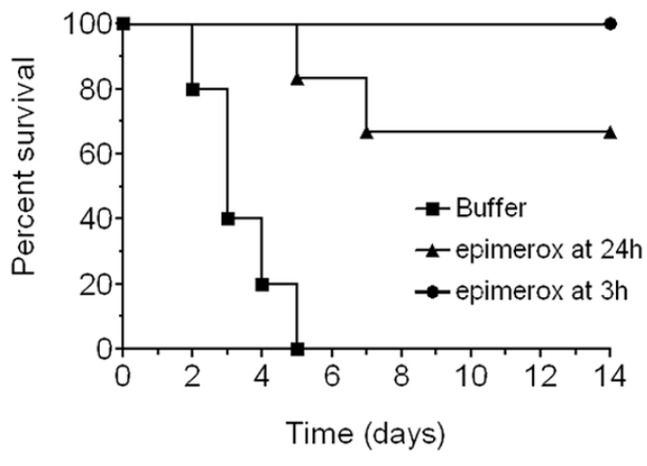

Br
OH
O
O
N
S
S
NH


b
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
0
2
4
6
8
10
12
14
Time (hours)
no epimerox
0.3 μM epimerox
3.0 μM epimerox


d
Growth inhibition (%control)
100
90
80
70
60
50
40
30
20
10
0
5μM epimerox
10μM epimerox
30μM epimerox
B. anthracis Sterne
B. anthracis ΔSterne
B. cereus 03BB87
B. thuringiensis Al-Hakam
B. cereus E33L
B. subtilis SL4
S. aureus ATCC BAA-42 (MRSA)
Streptococcus pyogenes D471 (GrAS)
Streptococcus agalactiae 090R (GrBS)
Streptococcus suis ATCC 43765
Enterococcus faecalis EF-1 (VRE)
Enterococcus faecium EFSK-2 (VRE)
Listeria monocytogenes HER 1184
Vibrio vulnificus-Env1
Vibrio vulnificus Clin1
Pseudomonas aeruginosa PA01
Acinetobacter baumannii ATCC BAA-747
Salmonella typhimurium LT2
Strains


c
no epimerox
epimerox
B. anthracis ΔSterne
Deltavision
merge
PlyGBD
Vanco.
S. aureus
RN4220
200 nm


e
1.2
1.0
0.8
0.6
0.4
0.2
0.0
OD600
0
2
4
6
8
10
12
Time (hours)
no epimerox
3.75 μM epimerox
7.5 μM epimerox
15.0 μM epimerox


f
100
80
60
40
20
0
Percent survival
0
2
4
6
8
10
12
14
Time (days)
Buffer
epimerox at 24h
epimerox at 3h

Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria

Ido Yosef[1], Miriam Manor[1], Ruth Kiro, and Udi Qimron[2]

Department of Clinical Microbiology and Immunology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

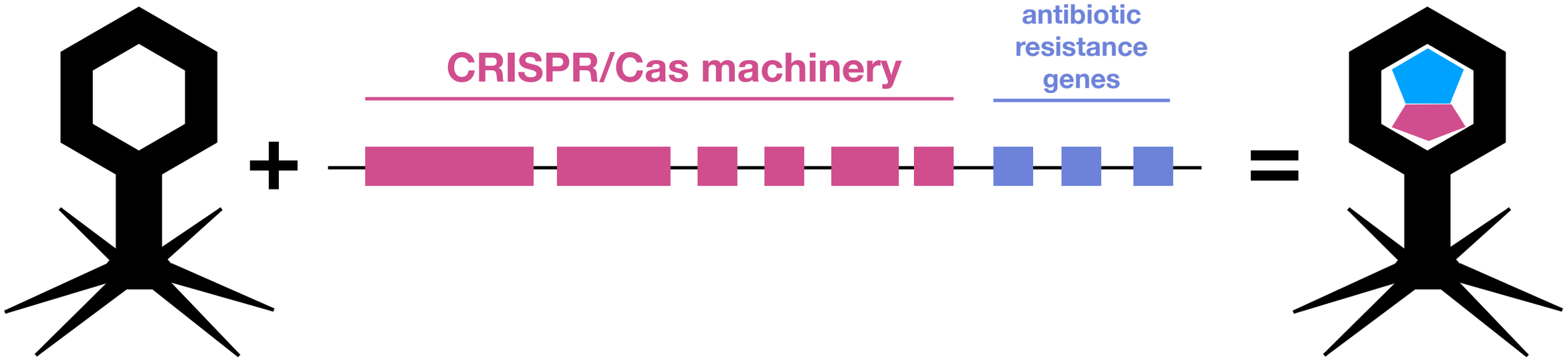
Edited by Jennifer A. Doudna, University of California, Berkeley, CA, and approved April 28, 2015 (received for review January 25, 2015)

The increasing threat of pathogen resistance to antibiotics requires the development of novel antimicrobial strategies. Here we present a proof of concept for a genetic strategy that aims to sensitize bacteria to antibiotics and selectively kill antibiotic-resistant bacteria. We use temperate phages to deliver a functional clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) system into the genome of antibiotic-resistant bacteria. The delivered CRISPR-Cas system destroys both antibiotic resistance-conferring plasmids and genetically modified lytic phages. This linkage between antibiotic sensitization and protection from lytic phages is a key feature of the strategy. It allows programming of lytic phages to kill only antibiotic-resistant bacteria while protecting antibiotic-sensitized bacteria. Phages designed according to this strategy may be used on hospital surfaces and hand sanitizers to facilitate replacement of antibiotic-resistant pathogens with sensitive ones.

CRISPR-Cas | positive selection | lysogenization | ex vivo treatment

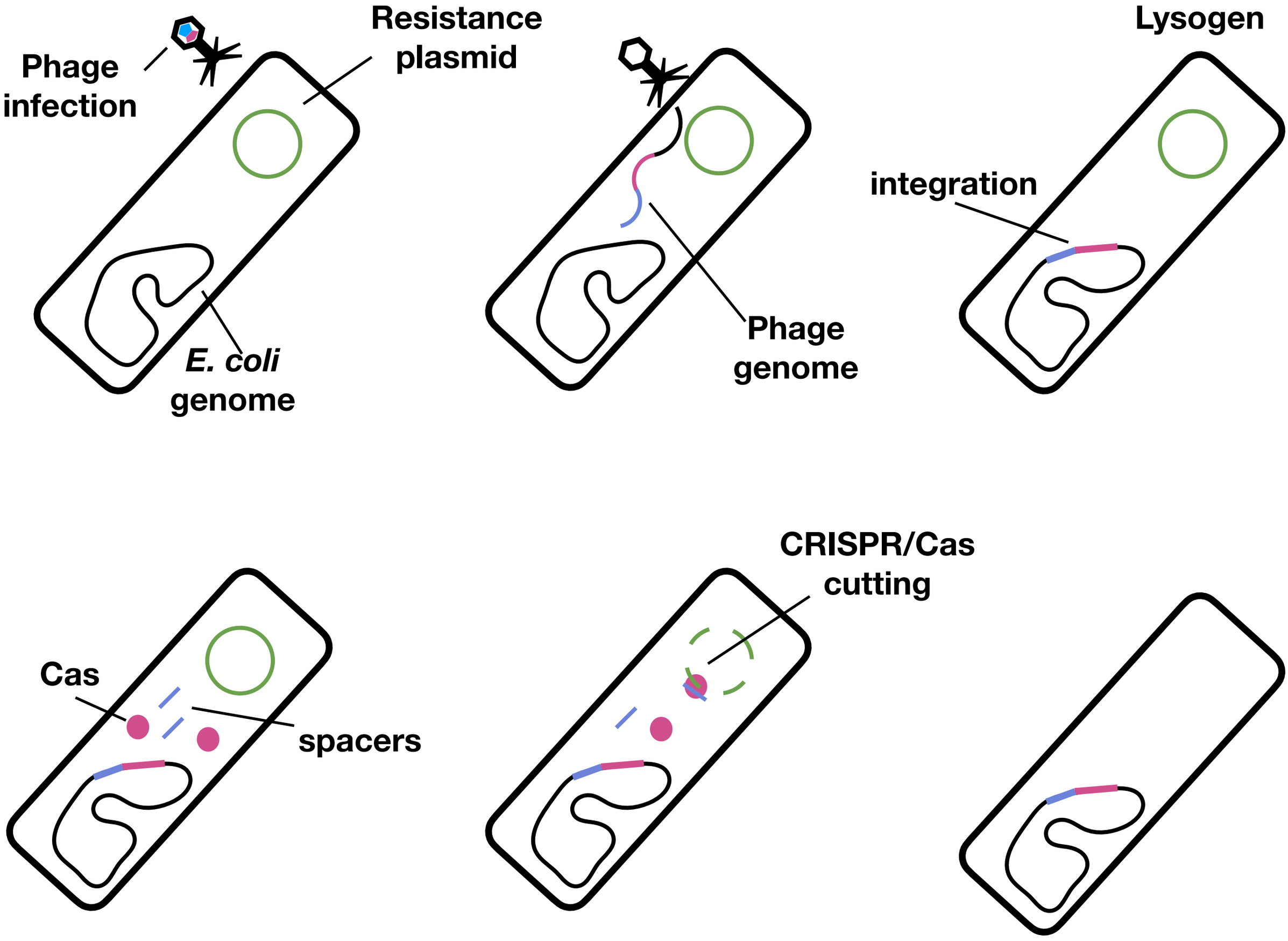
The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins have evolved in prokaryotes to protect against phage attack and undesired plasmid replication by targeting foreign DNA or RNA (1–3). These systems target nucleic acids, based on short sensitized pathogens would most likely fail due to escape mutants that are selected by the antibiotics.

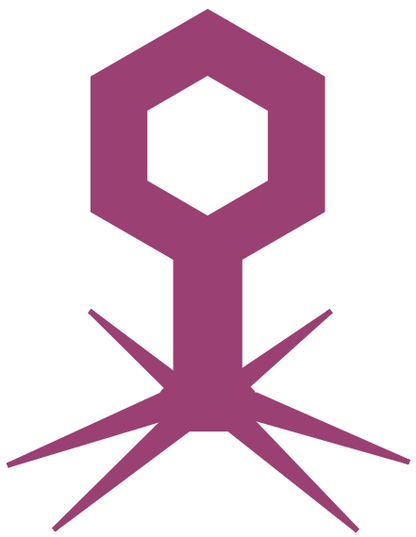
Here we demonstrate a strategy to counteract the emerging threat of antibiotic-resistant bacteria that evades the above shortcomings. Instead of directly killing the pathogens, we propose to sensitize the pathogens on surfaces or in the human skin flora while concomitantly enriching for these sensitized populations. Patients infected by these antibiotic-sensitive bacteria would thus be treatable by traditional antibiotics. In this strategy, the CRISPR-Cas system is used to destroy specific DNAs that confer antibiotic resistance and to concurrently confer a selective advantage to antibiotic-sensitive bacteria by virtue of resistance to lytic phages. The selective advantage enables to efficiently displace populations of nonsensitized bacteria by killing them with lytic phages. In contrast to conventional phage therapy, this approach does not require administration of phages into the host's tissues. In addition, it does not aim to directly kill treated bacteria but rather to sensitize them to antibiotics and to kill the nonsensitized bacteria. Therefore, there is no counterselection against the sensitization. The strategy relies on CRISPR spacers that can be rationally designed to target any DNA sequence, including those that encode resistance genes and lytic phages. It thus allows genetically linking a trait that is beneficial to the bacteria (i.e., spacers protecting from lytic phage) with a trait that reverses drug resistance



Lysogenic phage

Lysogenic +CRISPR/Cas for antibiotic resistance


Phage infection
Resistance plasmid
E. coli genome
Phage genome
Lysogen
integration
Cas
spacers
CRISPR/Cas cutting

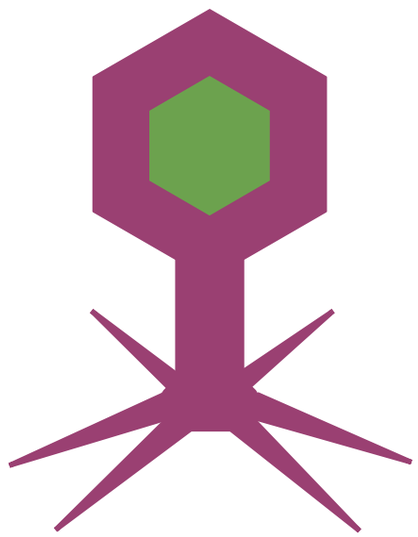


Lytic
phage

+

Antibiotic spacers identical to
lysogenic phage

=



Lytic phage +
antibiotic spacers

Lysogen

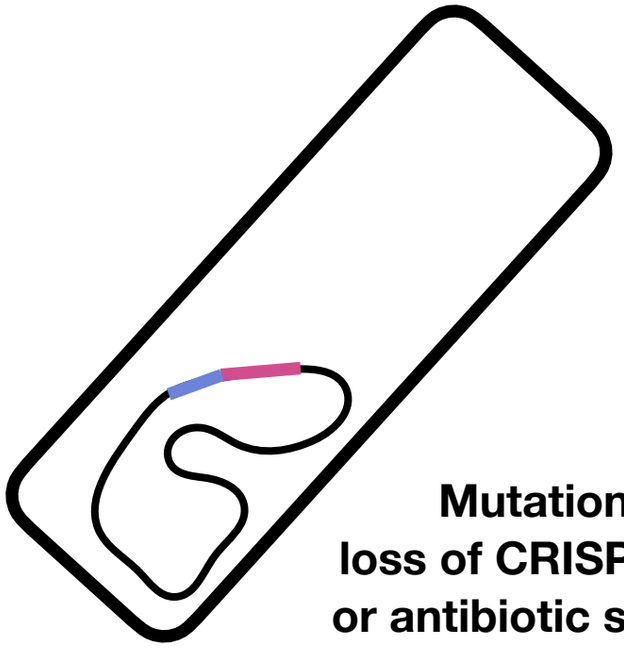
CRISPR/Cas
cutting of lytic
phage genome

Lytic phage
immune *E. coli*
with no antibiotic
resistance

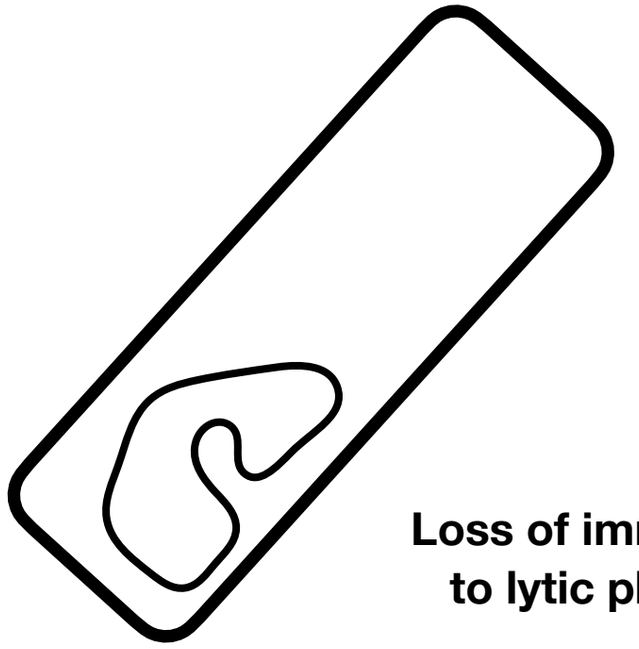
Non-lysogen

integration &
production of lytic
phage

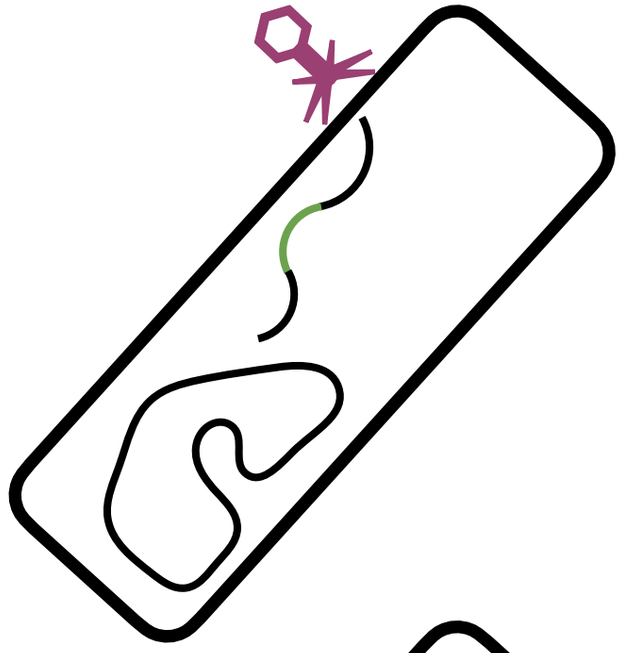
Lysed *E. coli*
release of
lytic phage

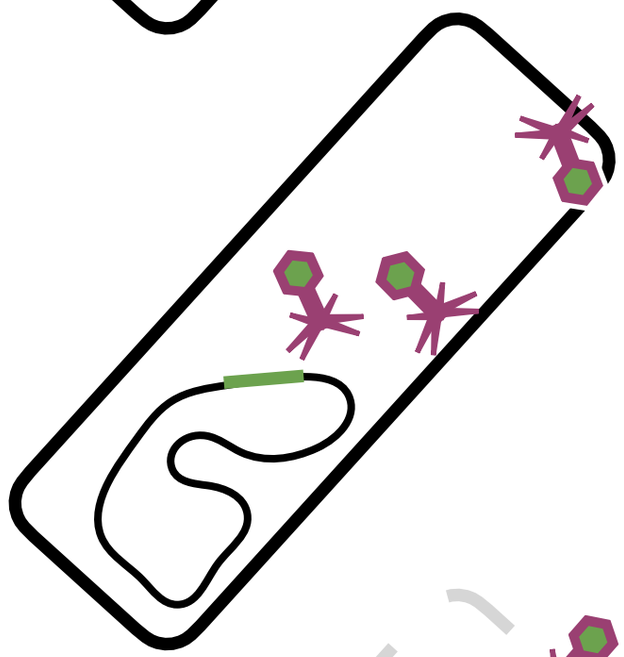


Mutational
loss of CRISPR/Cas
or antibiotic spacers



Loss of immunity
to lytic phage

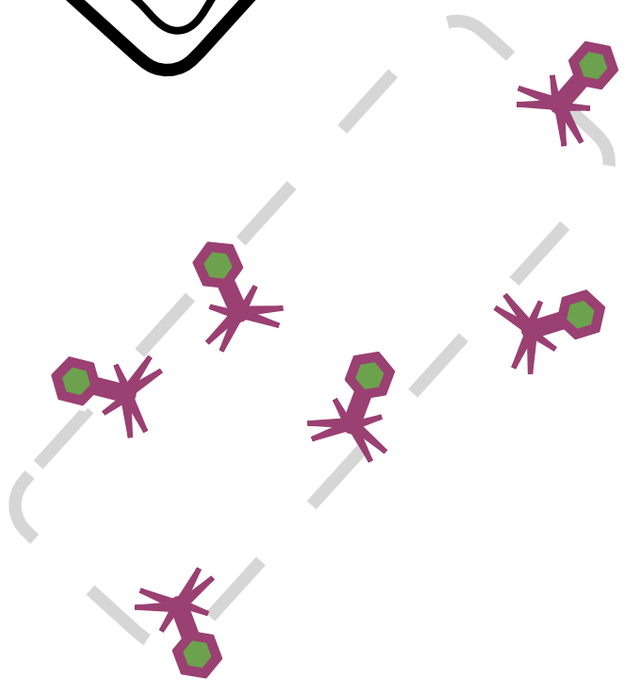




Linking of loss of antibiotic resistance to a selective advantage - resistance to engineered lytic phage.

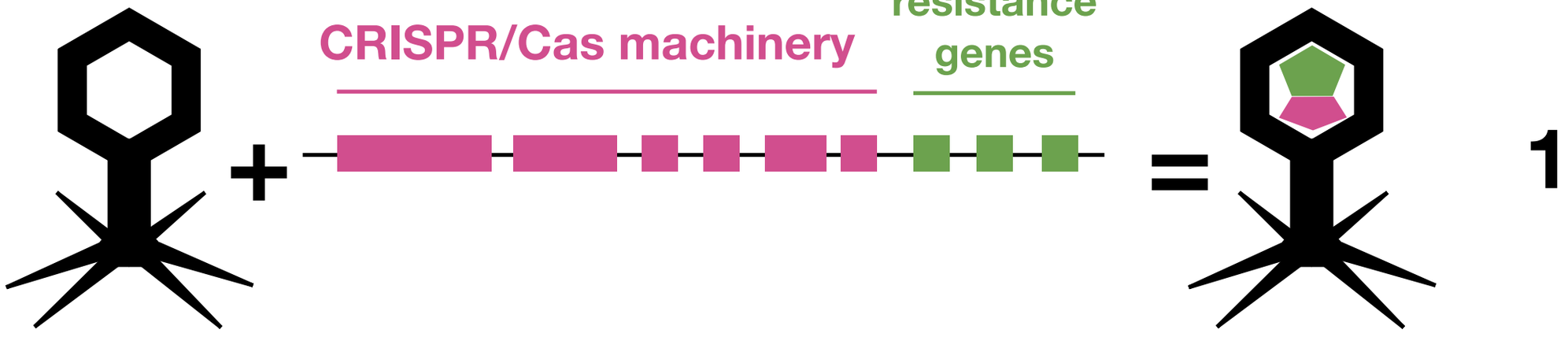
Lysogens can't lose CRISPR/Cas system that selects against antibiotic resistance, without losing immunity to engineered phage.

Immunity is only to engineered phage - maintains normal selection of *E. coli* populations - but selects against antibiotic resistance



Spacers for antibiotic resistance genes

CRISPR/Cas machinery



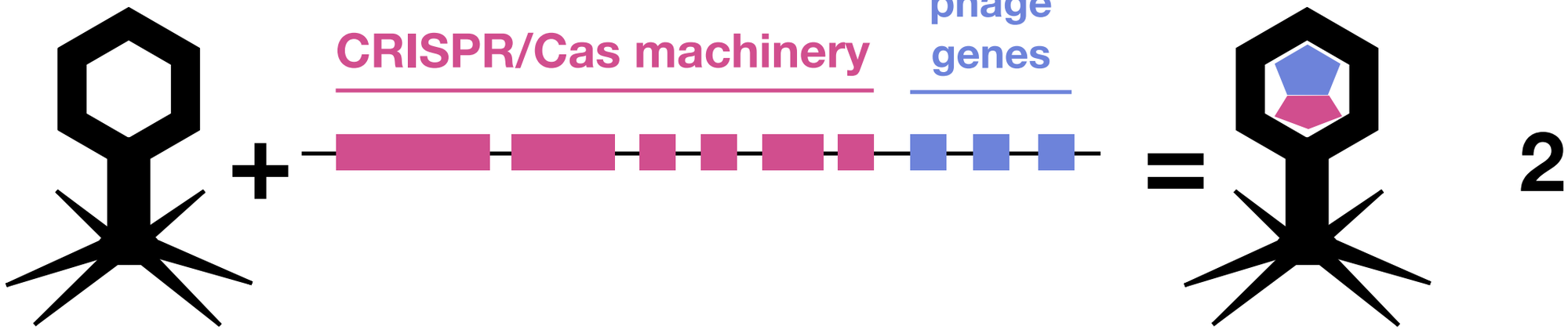
Lysogenic phage

Lysogenic +CRISPR/Cas for antibiotic resistance

1

Spacers for lytic phage genes

CRISPR/Cas machinery

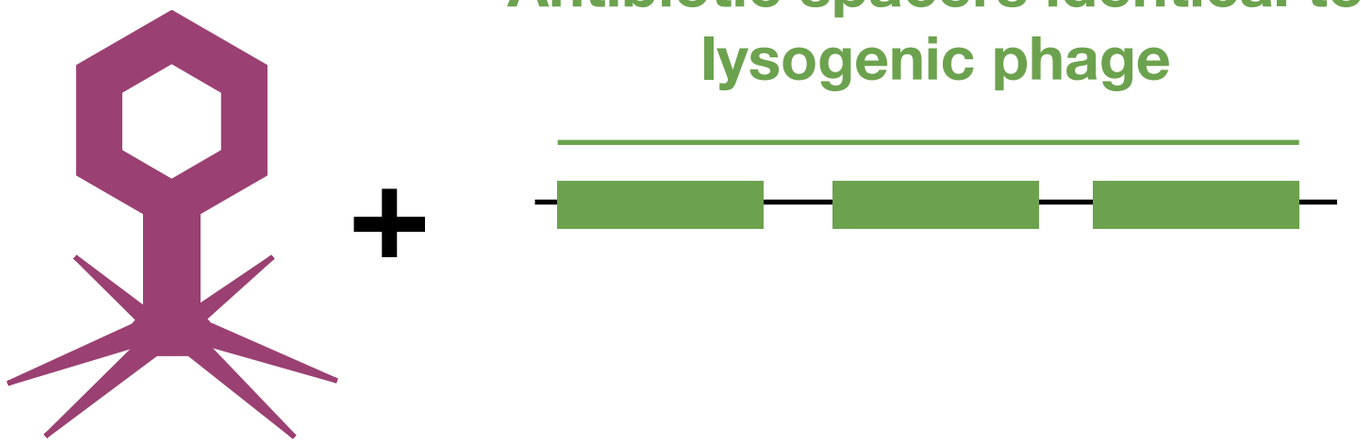


Lysogenic phage

Lysogenic +CRISPR/Cas for lytic phage

2

Antibiotic spacers identical to lysogenic phage



Lytic phage

Lytic phage + antibiotic spacers

3

Lytic phage spacers identical to lysogenic phage

Lytic phage

Lytic phage + lytic phage spacers

4

Question 4.

In order to select for *E. coli* populations that have no antibiotic resistance, which combination of engineered phages was used?