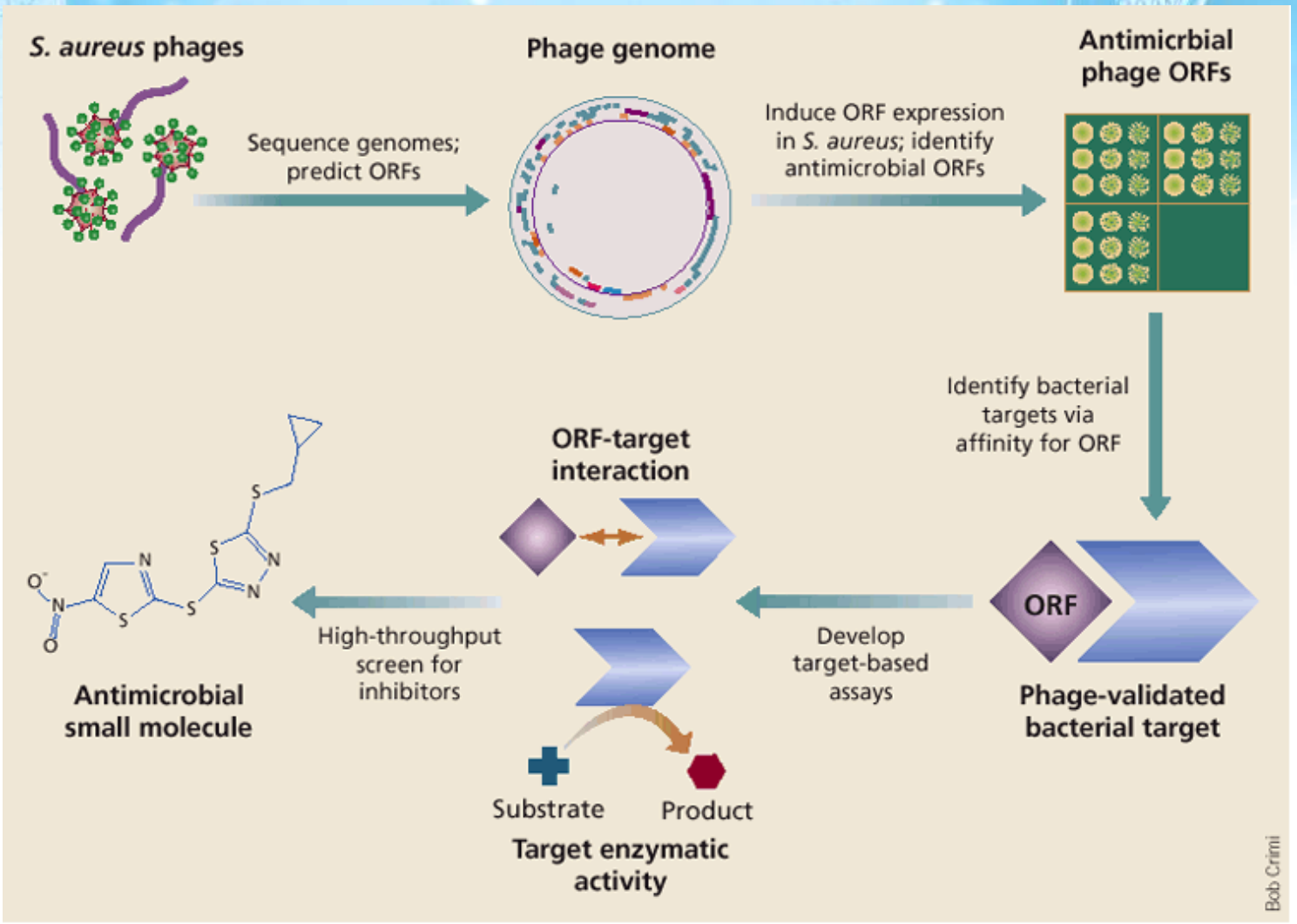
Phage-Inspired Discovery of Small-Molecule Antibiotics

May 18, 2006

Targanta Therapeutics

Phage-Inspired Drug Discovery Approach



Liu et al., 2004. Nature Biotech. 22:185

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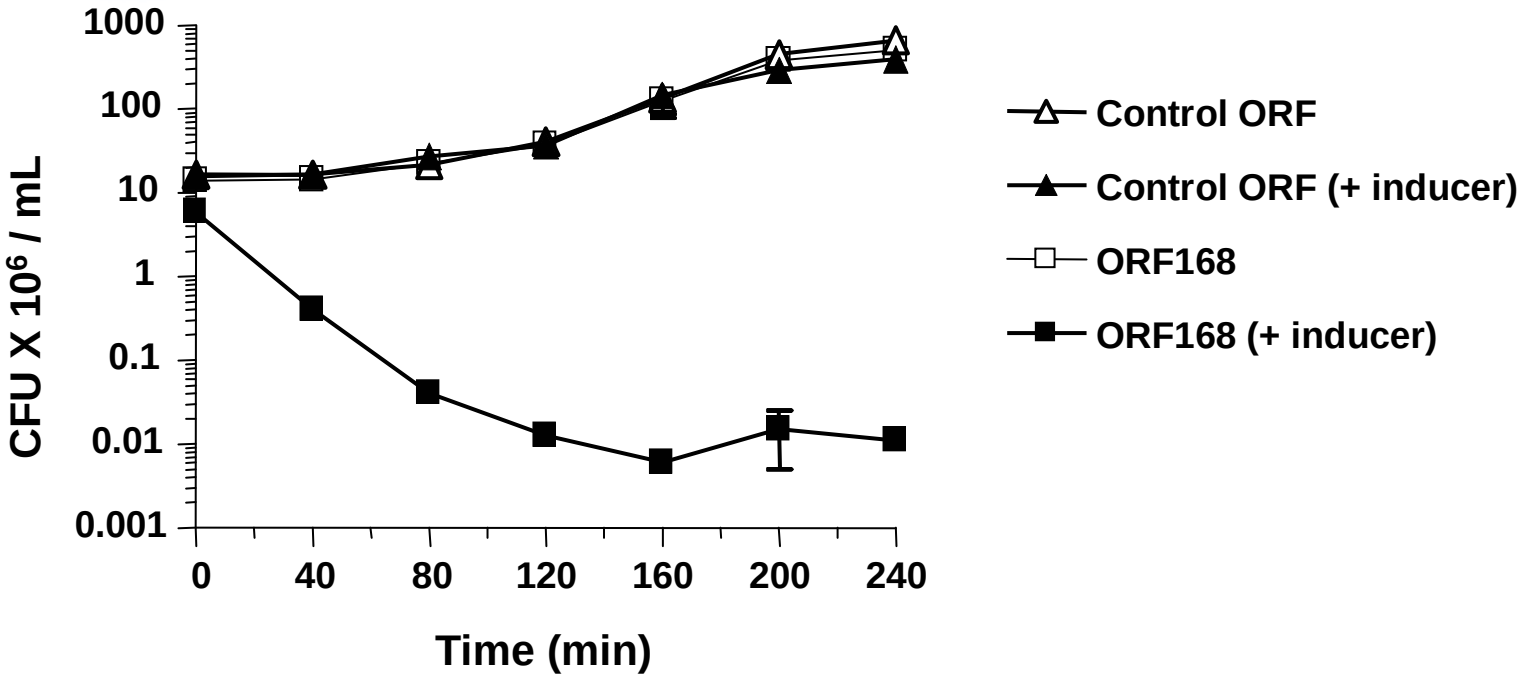
Update On Recent Data

- Target identification and validation by phage polypeptides
- Identification of small molecule inhibitors
- Optimization of inhibitors via medicinal chemistry
- Two examples:
  - *S. aureus* DNA replication target: DNA polymerase β subunit
  - *S. aureus* transcription target: primary sigma factor RpoD

S. aureus DNA replication target: DNA polymerase β subunit

Expression of ORF168 in *S. aureus* is Bactericidal

- Dot screening → phage open reading frames that inhibit growth when expressed within *S. aureus*
- Broth assay → rapid killing kinetics of selected phage ORFs:



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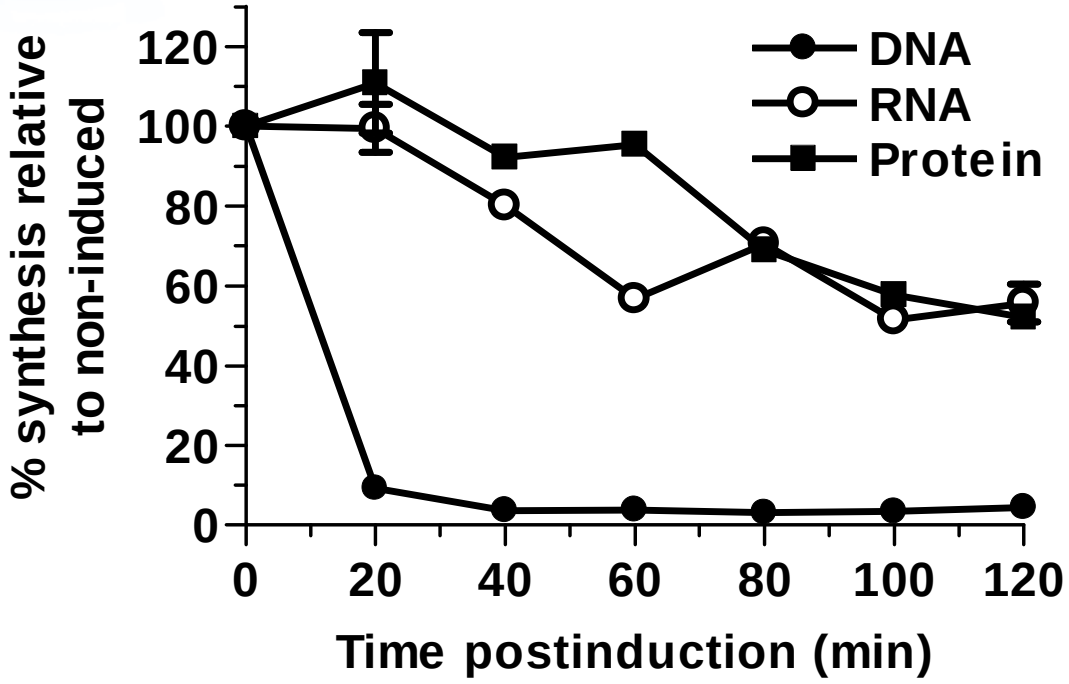
Expression of ORF168 in *S. aureus* Selectively Inhibits DNA Synthesis

• Macromolecular synthesis assay in *S. aureus* → selectivity of inhibition:



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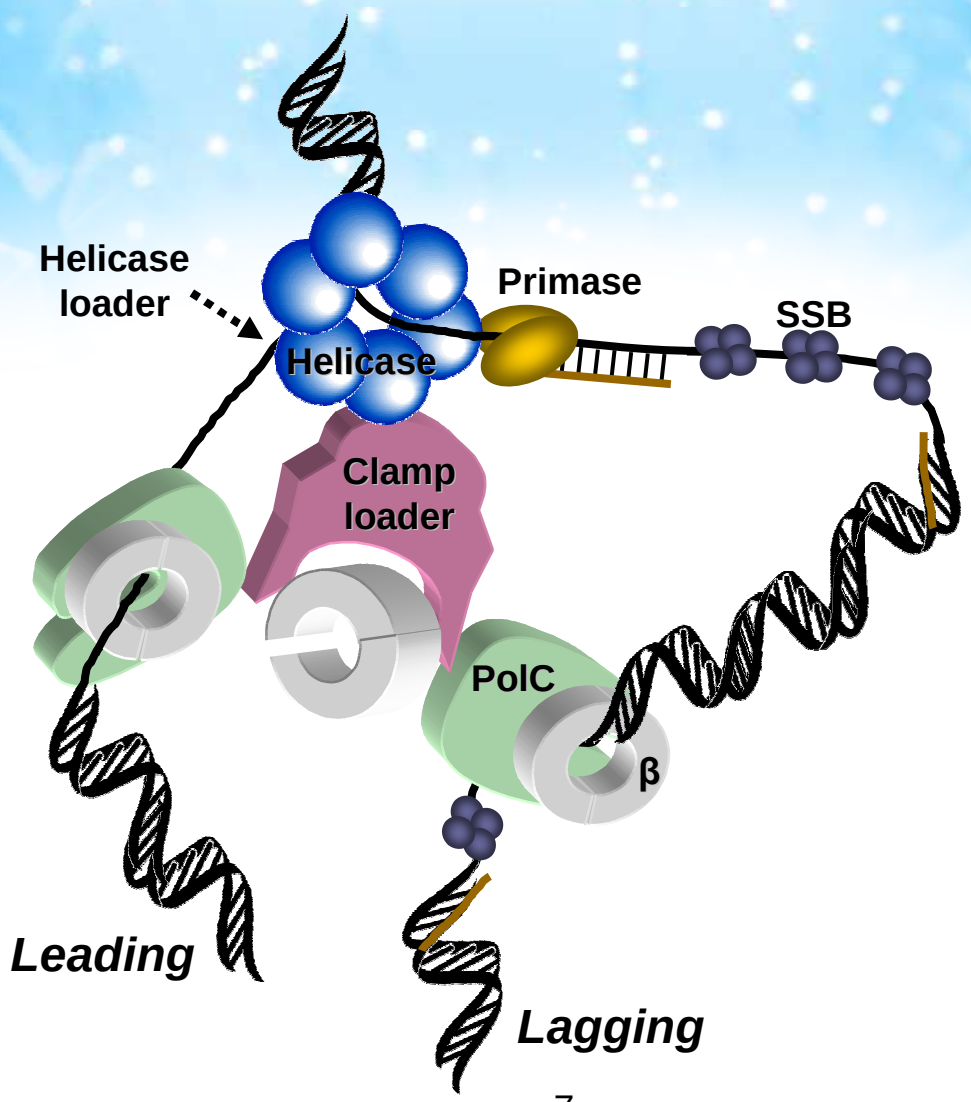
The Bacterial DNA Replication Machinery Offers Essential, Under-Exploited Targets



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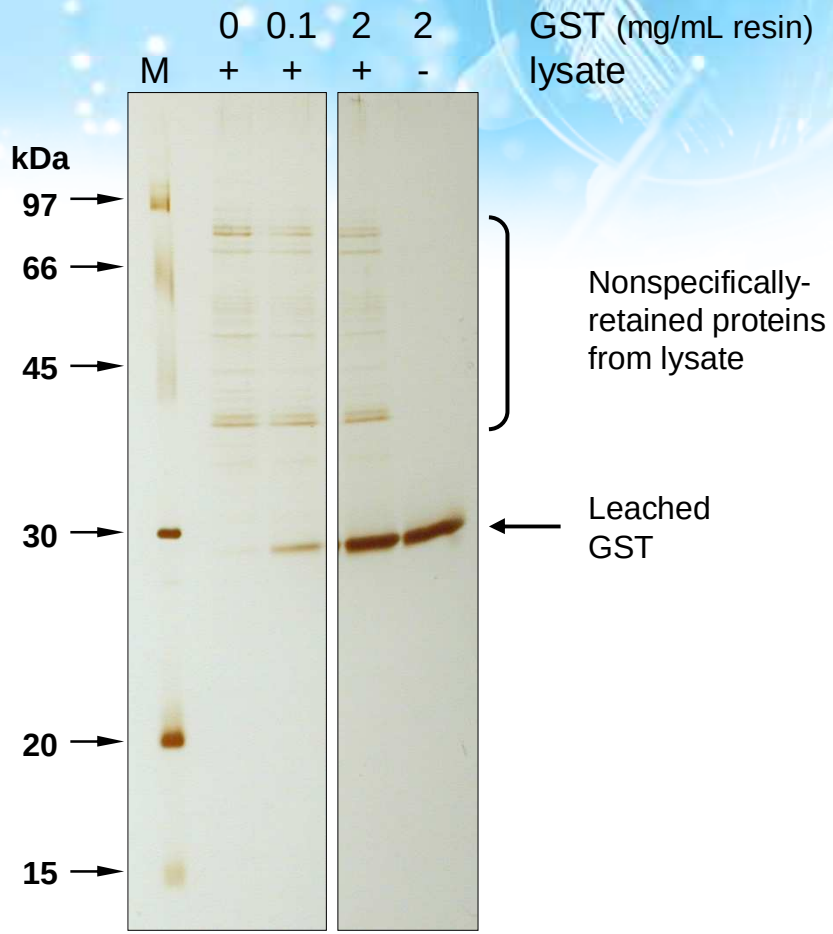
ORF Affinity Chromatography to Identify Cellular Targets of Phage Polypeptides

- Immobilize GST-ORF168 (or GST as control) to column
- Apply *S. aureus* extract (or buffer as control)
- Wash, elute retained proteins with 1% SDS
- SDS-PAGE, silver staining
- Excise bands of interest and digest with trypsin
- Determine masses of tryptic peptides by MALDI-ToF mass spectrometry

ORF Affinity Chromatography to Identify Cellular Targets of Phage Polypeptides



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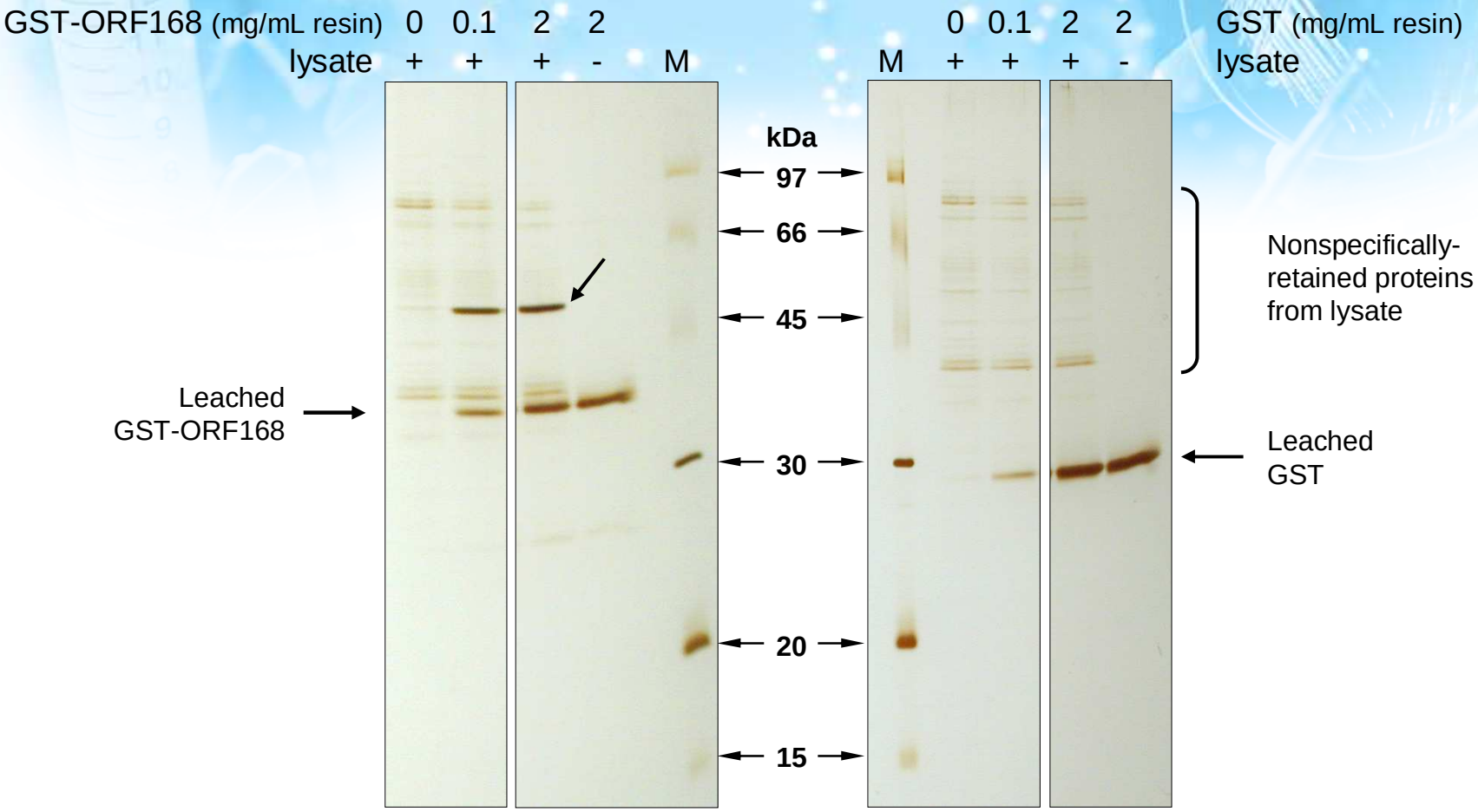
ORF168 Affinity Chromatography Identifies a ~45 kDa Prey Protein from *S. aureus* lysate



- *One candidate interacting protein was eluted specifically from the ORF168 affinity column*

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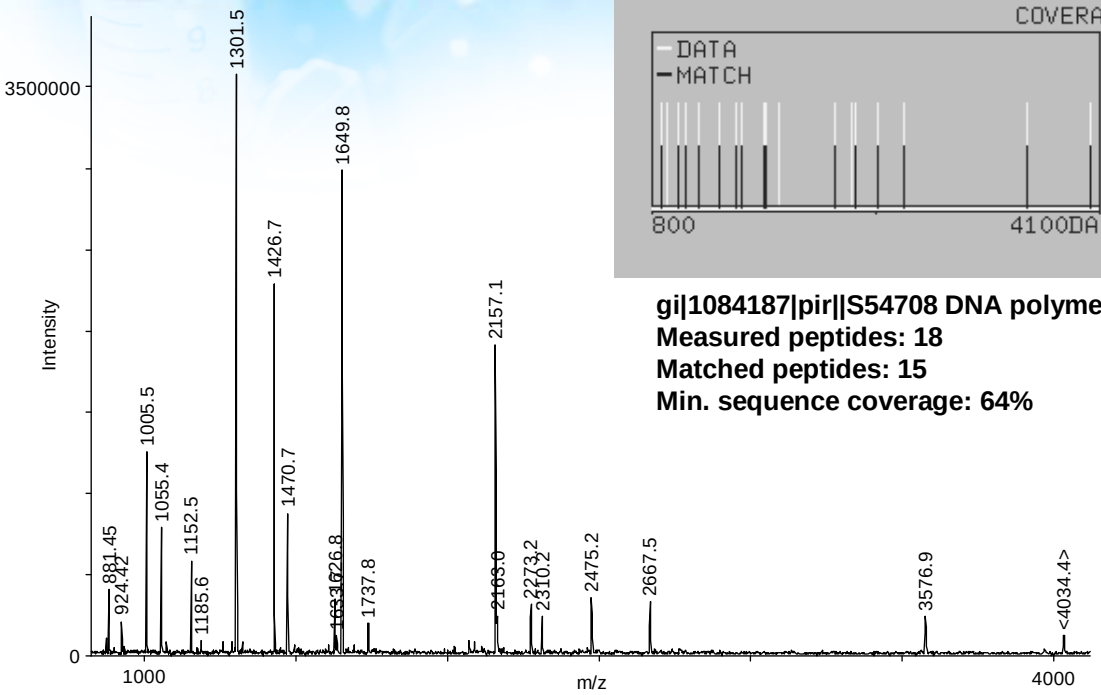
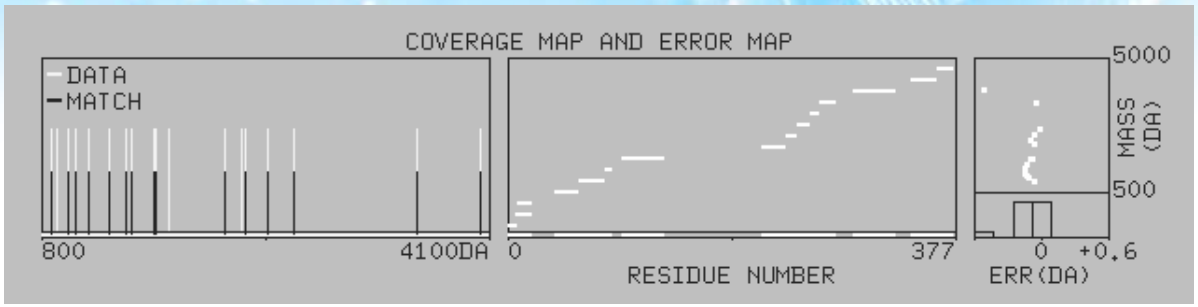
Tryptic Fingerprint Identifies *S. aureus* DNA Pol β as the Target of ORF168





gi|1084187|pir||S54708 DNA polymerase III beta chain - *Staphylococcus aureus*
Measured peptides: 18
Matched peptides: 15
Min. sequence coverage: 64%

- *Tryptic peptide mass spectrum identifies the target of ORF168 as the β subunit of S. aureus DNA Polymerase C*

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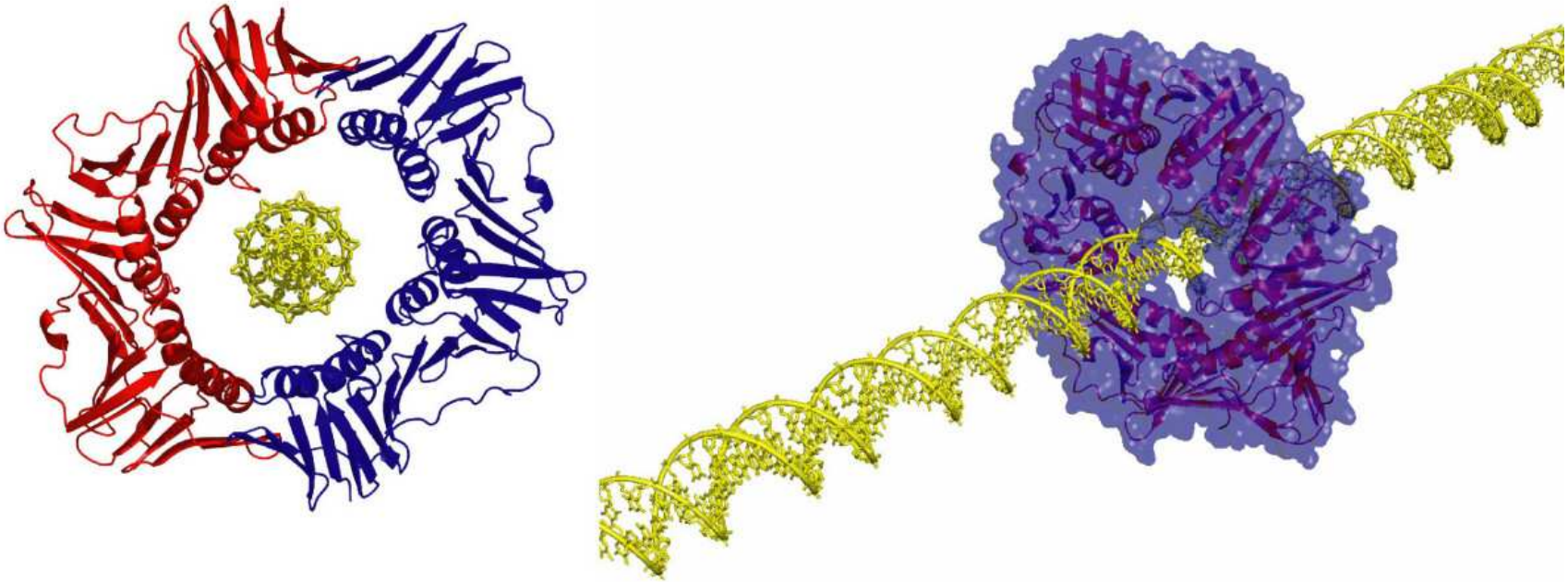
Structure of DNA Pol β from *Streptococcus pyogenes*

- Three-domain structure in protomer
- Homodimer shares overall topology with other bacterial DNA sliding clamps despite sequence divergence (e.g. 23% a.a. identity with *E. coli* β)



Argiriadi et al., 2006. BMC Struct. Biol. 6:2

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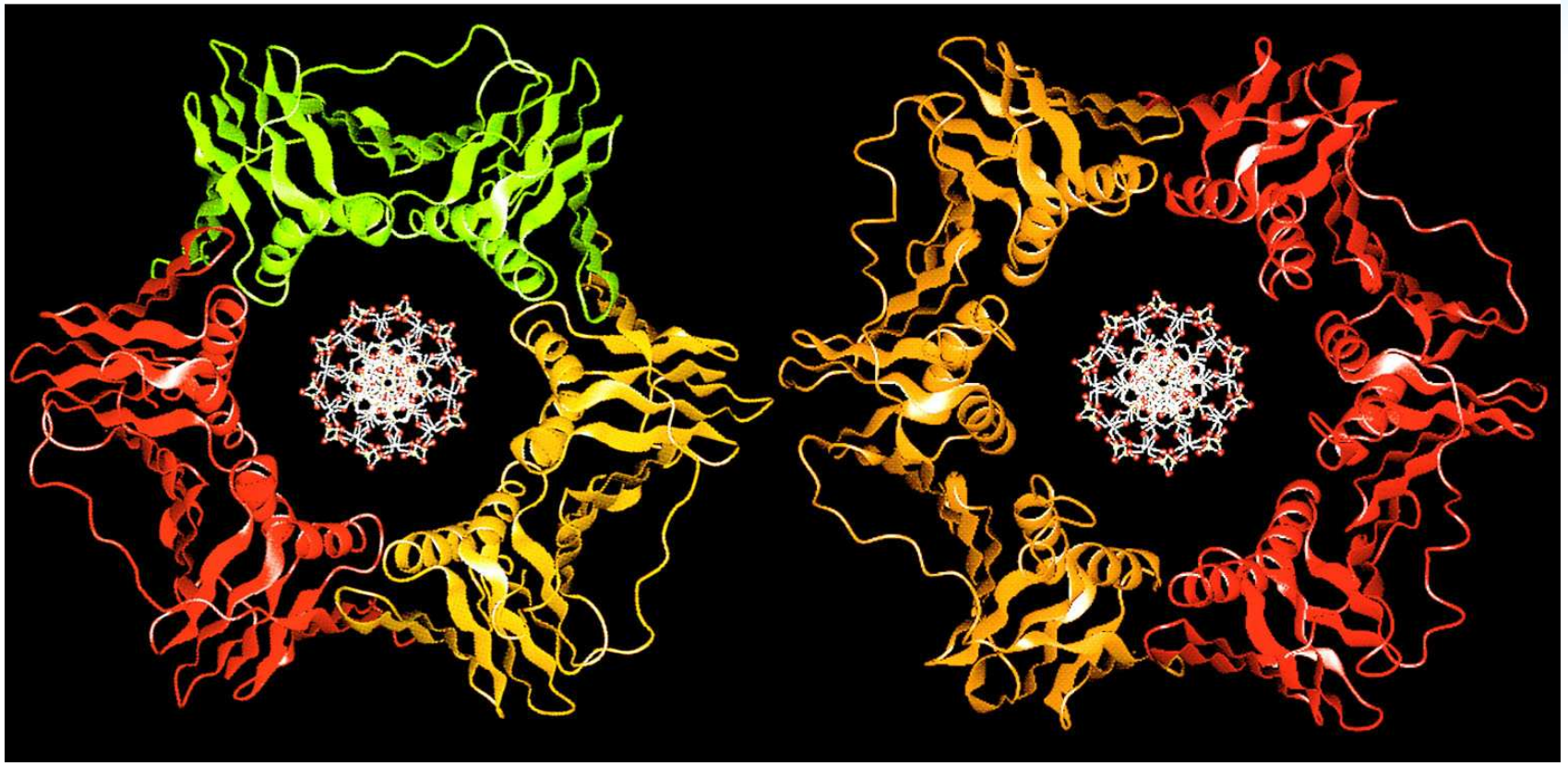
DNA Sliding Clamps Share Mechanistic Similarity Despite Divergence in Sequence

Yeast PCNA

E. coli β



Krishna et al., 1994. Cell 79:1233

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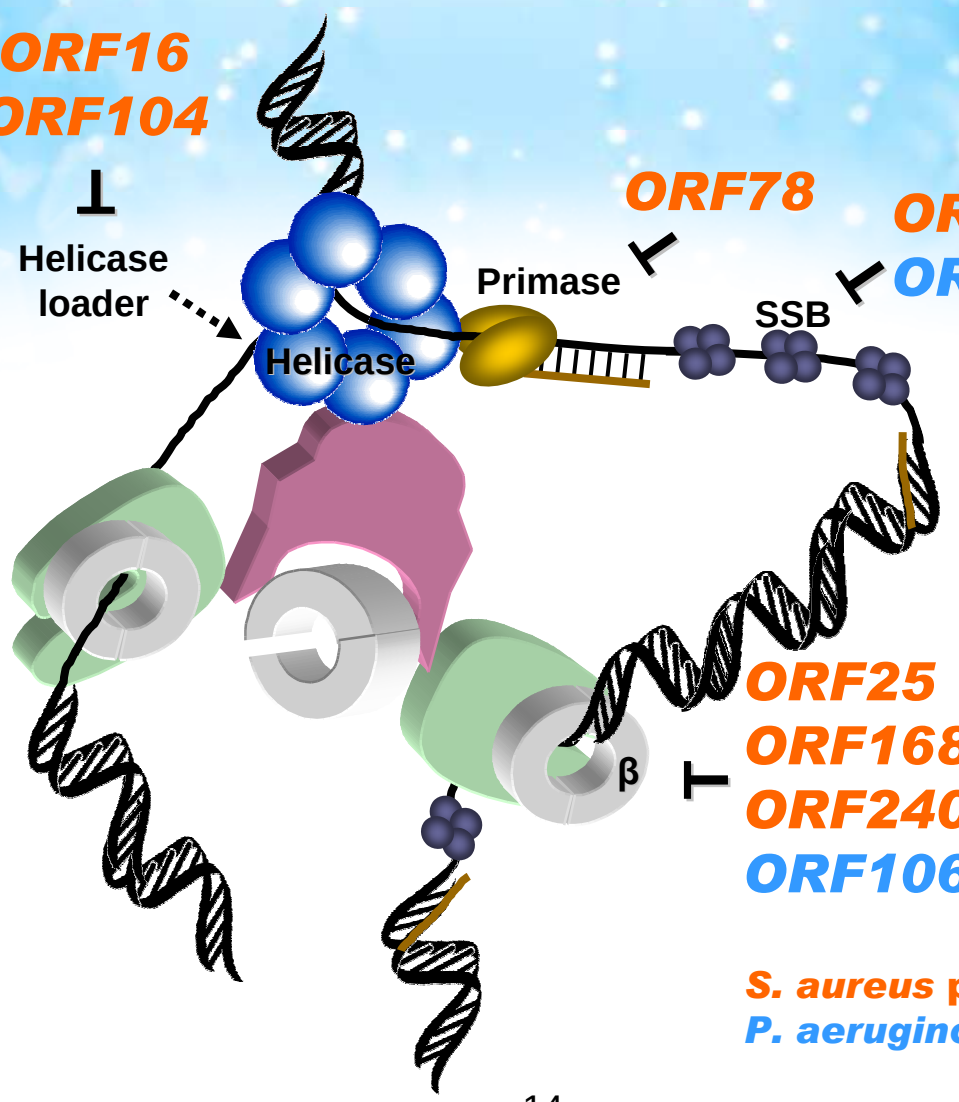
Affinity Chromatography Identifies Replication Machinery Targets for Phage Polypeptides



***S. aureus* phage polypeptide**
***P. aeruginosa* phage polypeptide**

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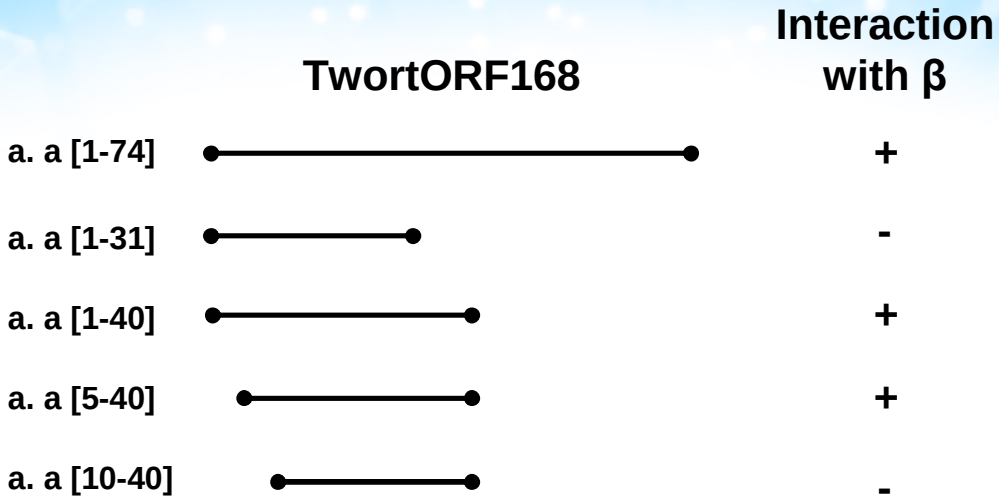
Region of Highest Sequence Similarity in ORF168 and ORF240 is Required for Binding to β

- Yeast two-hybrid assay with truncated ORF168 and full-length β → identify minimal interacting domain of phage polypeptide



Minimal domain of interaction

```
TwortORF168 ----MLFFKEKFYNELSYYRGGHKDLESMFELALEYIEKLEEEDEQQVTDYENAMEEELRDAVDVIESQLEIIKDIVR
  G1ORF240 MVIPSIKAQNKFKNELEYYKQGHISESKMLELAFDYIQELEQNNE-----YVTNLLEEERYGE--------------
                    KF NEL YY  GH     M ELA  YI  LE   E     Y     EE R
```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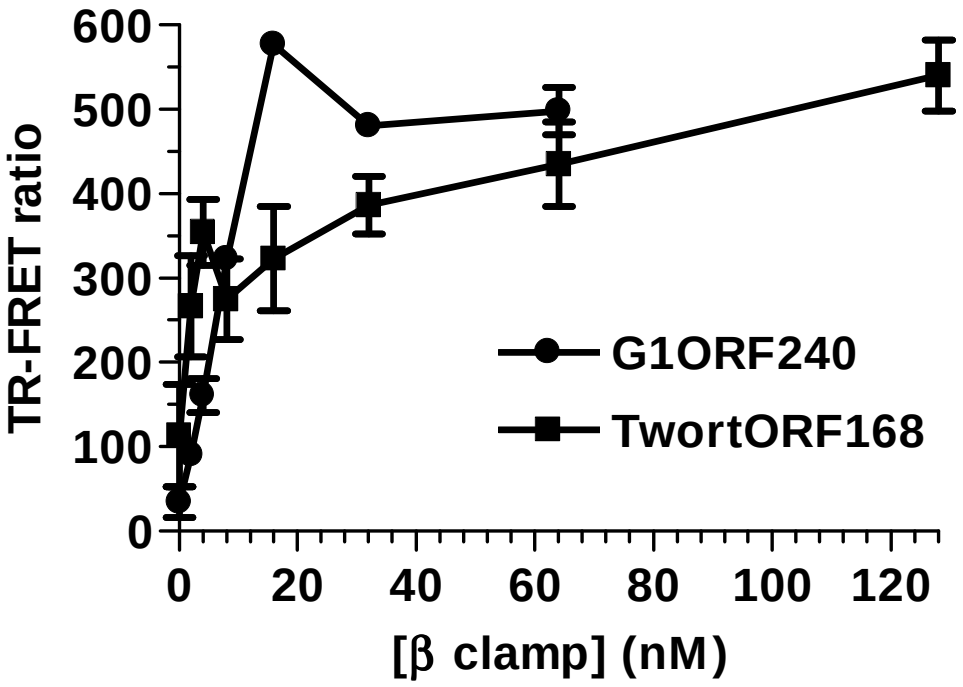
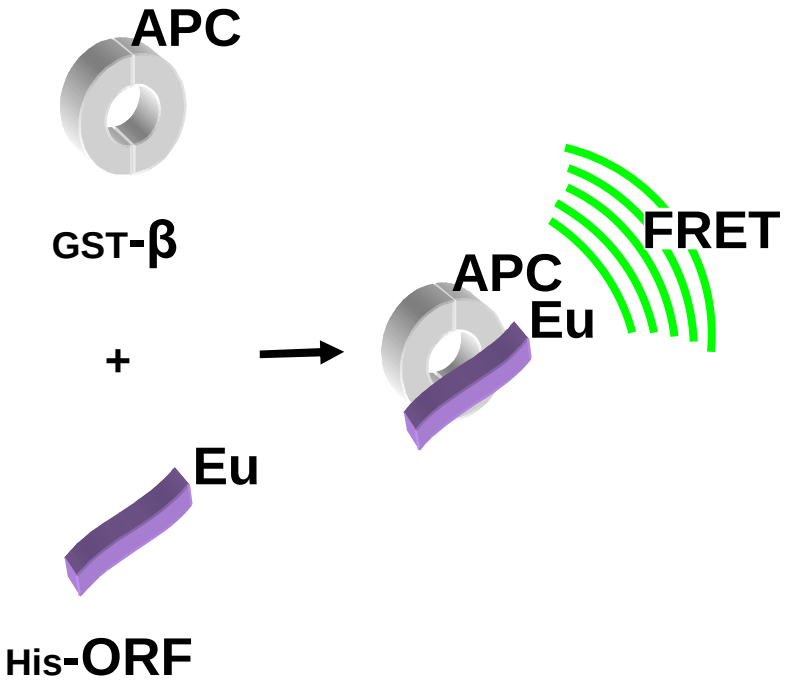
Phage Polypeptides ORF240 and ORF168 Bind to β in Solution

- TR-FRET fluorescence assay → confirm the interaction in solution:





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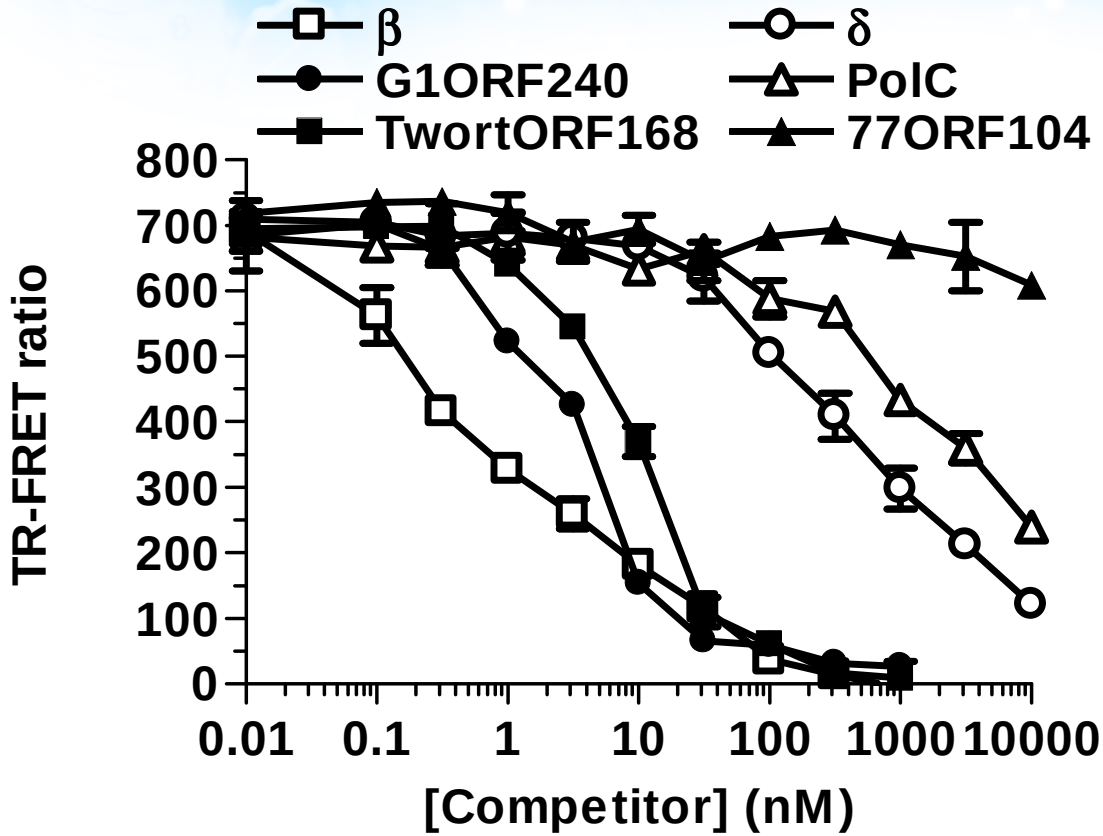
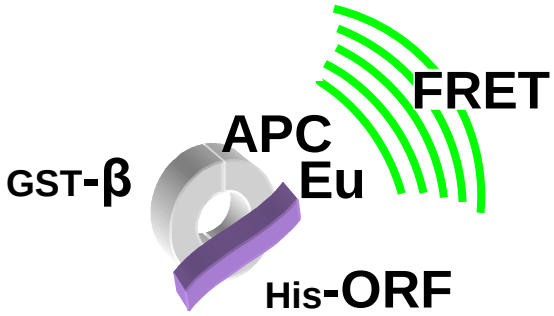
Phage Polypeptide Binding Site on β Appears to be Shared by Replicase Components

- TR-FRET fluorescence assay → study competitors of the interaction:





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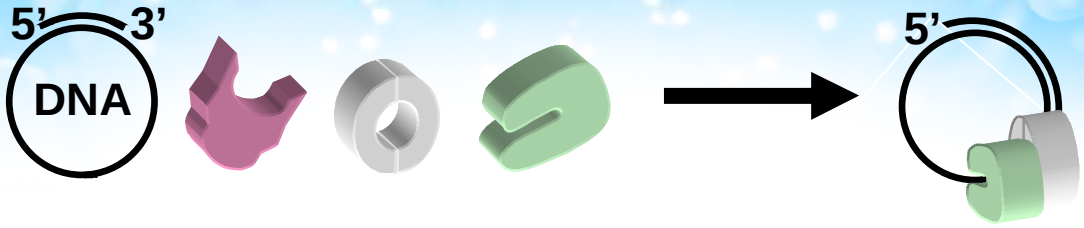
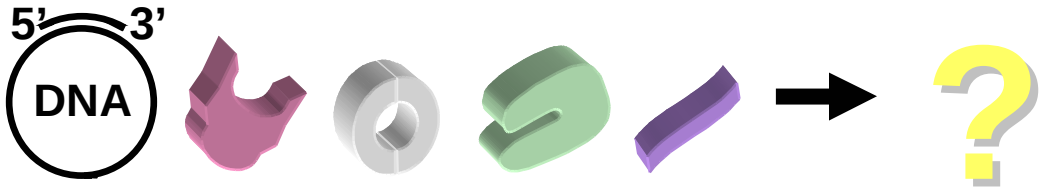
Does Phage Polypeptide Binding to β have a Functional Consequence?



Clamp loader **β** **PolC** **Processive DNA replication**



Clamp loader **β** **PolC** **ORF**

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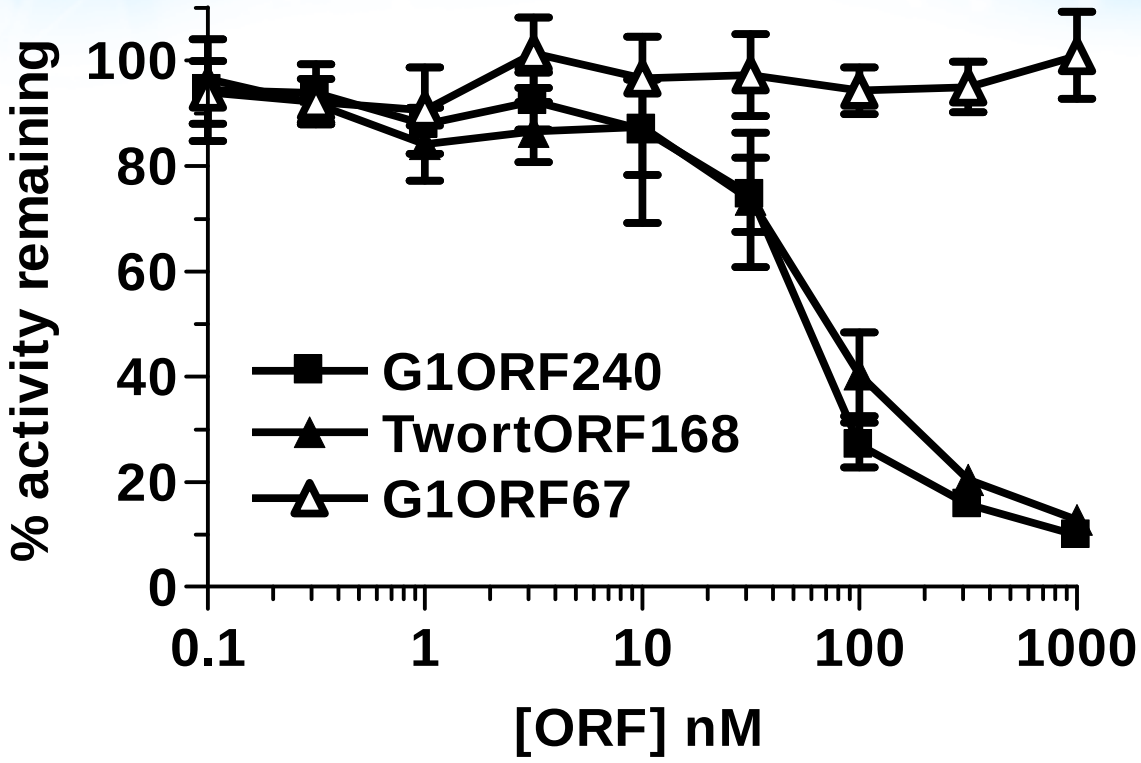
β-binding Phage Polypeptides Inhibit the *S. aureus* DNA Replicase *in vitro*

• Plate-based assay with reconstituted replicase → study effect of phage polypeptides on processive DNA synthesis *in vitro*:



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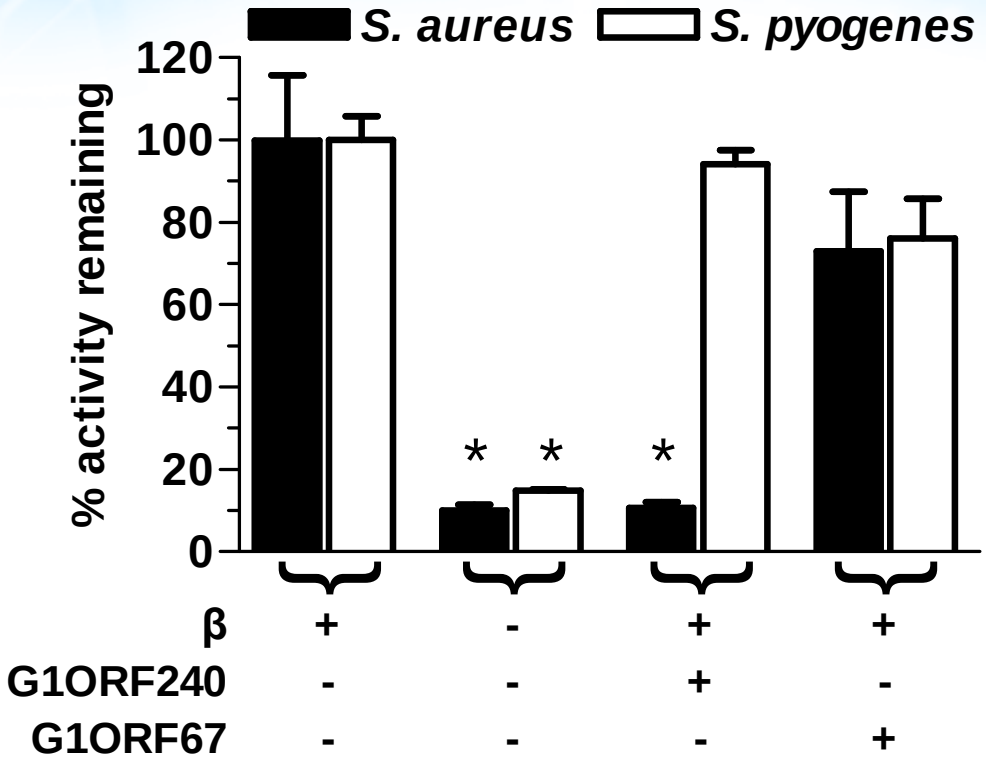
Inhibition of DNA Synthesis *In vitro* by ORF240 is Selective for *S. aureus*

• Plate-based assay with replicases from *S. aureus* and *S. pyogenes* → study selectivity of phage polypeptide inhibition of DNA synthesis *in vitro*:



***P* < 0.05**

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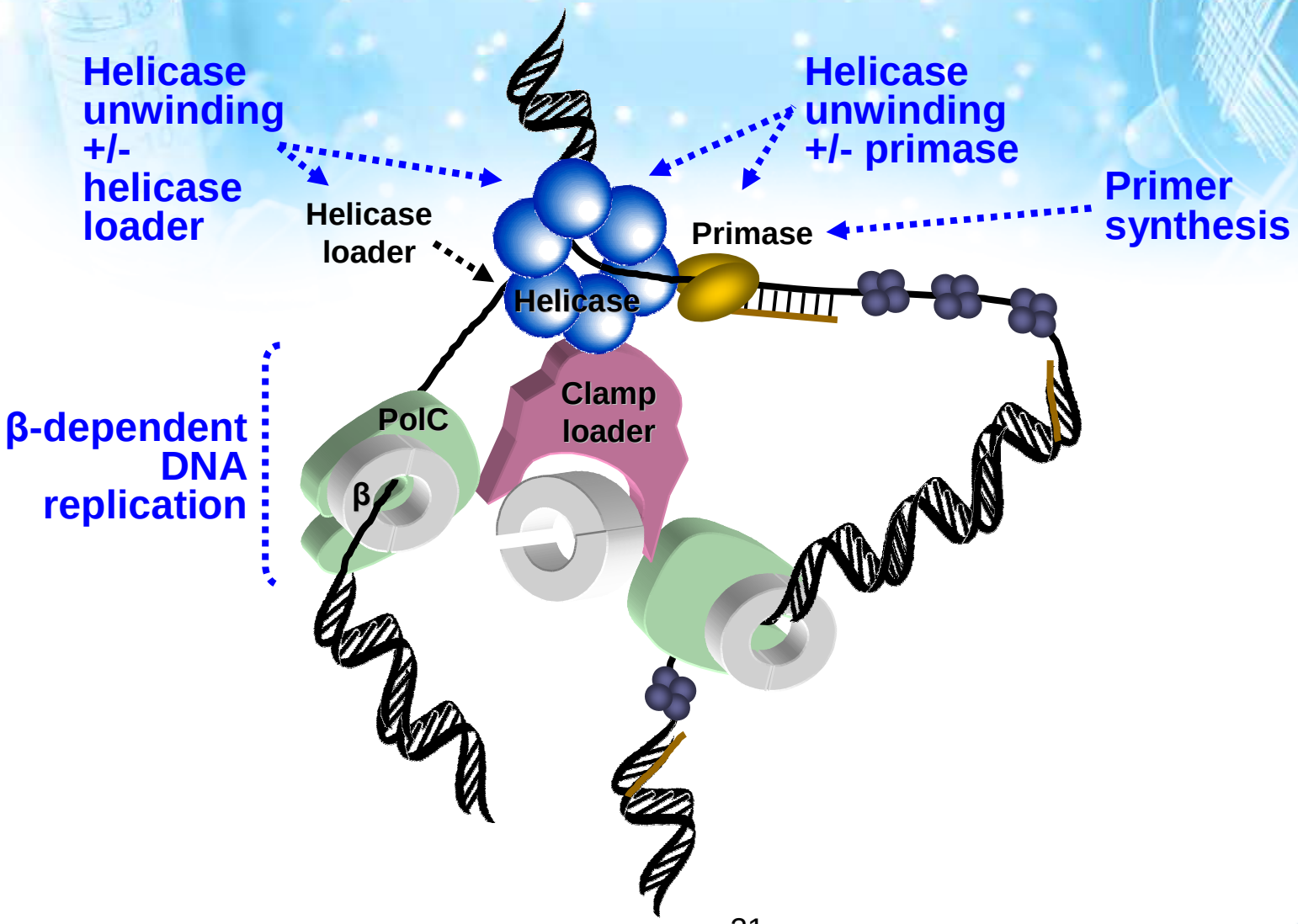
Enzyme-Based Screens for Small Molecule Inhibitors Exploit Essential Activities of the Replicase



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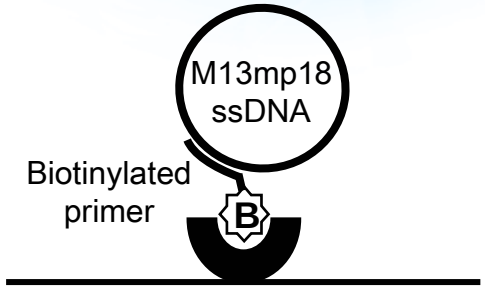
Screening for Small-molecule Inhibitors of the *S. aureus* Replicase



Streptavidin Flashplate

Low CPM

- SSB
- Clamp loader
- β
- PolC (polymerase)
- [^{3}H]-dTTP
- dNTPs

- RT, 1h
- EDTA

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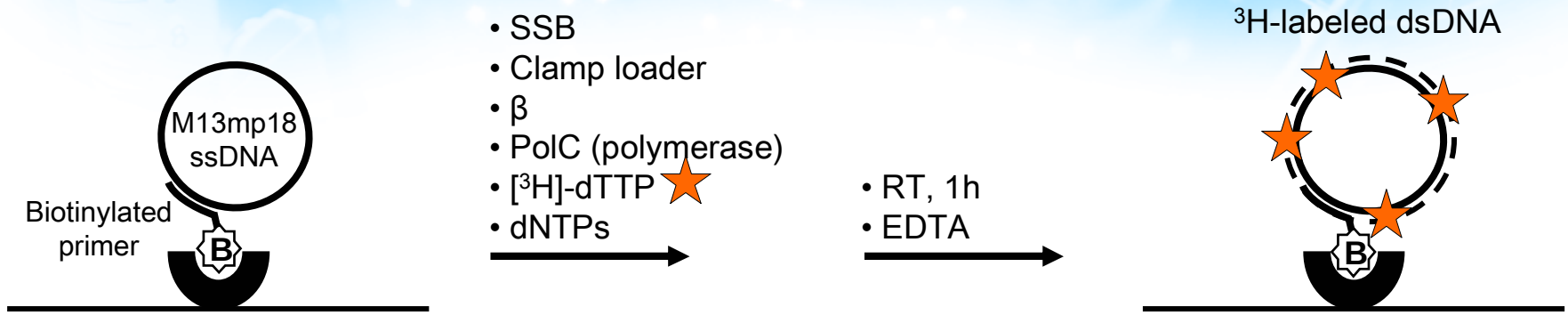
Screening for Small-molecule Inhibitors of the *S. aureus* Replicase



Streptavidin Flashplate

Low CPM

High CPM

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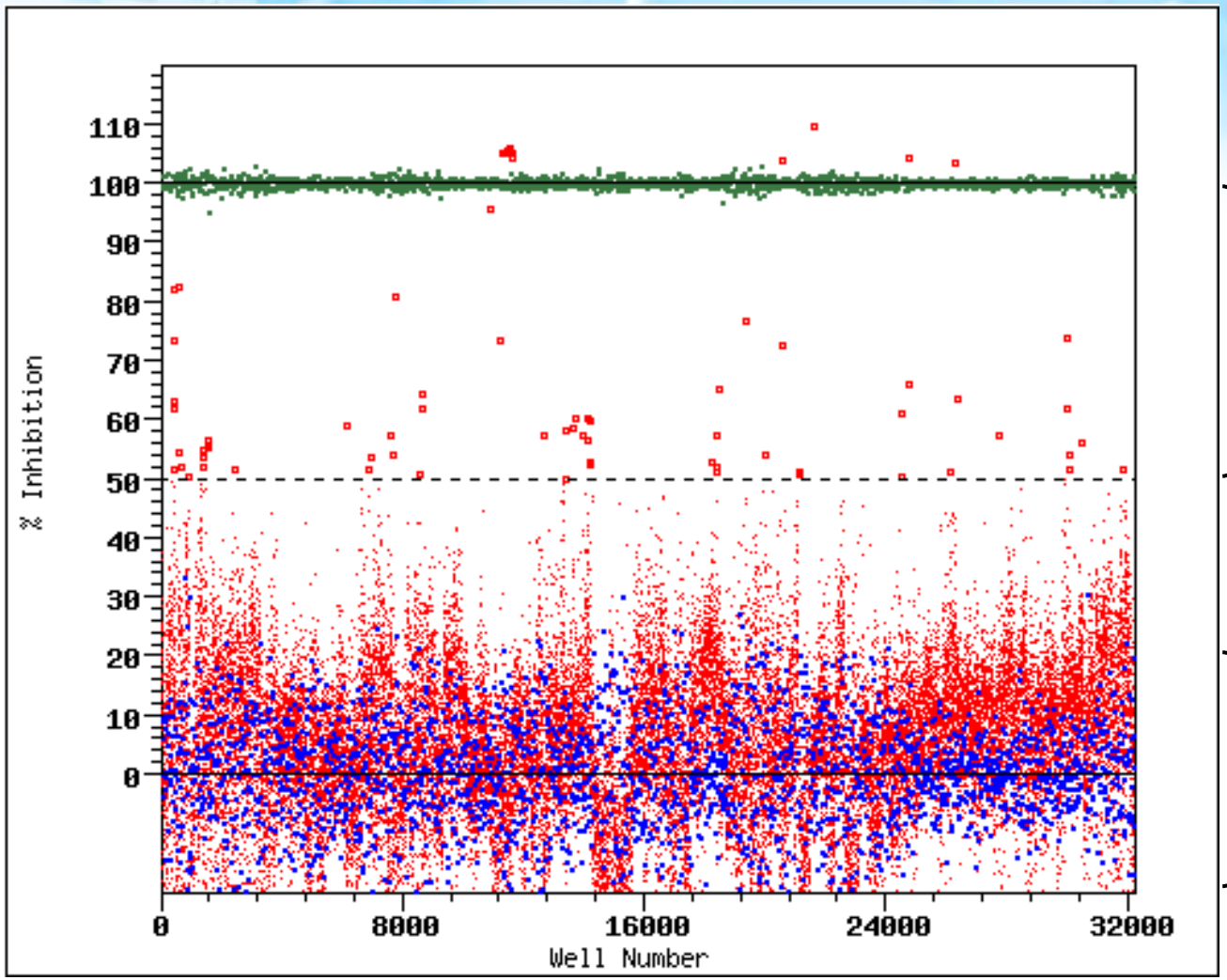
Screening for Small-molecule Inhibitors of the *S. aureus* Replicase

- HTS of 126,400 small-molecule compounds (n=4/well):



Positive controls

Actives

50% inhibition threshold (~3*s.d.)

Compounds, Negative controls

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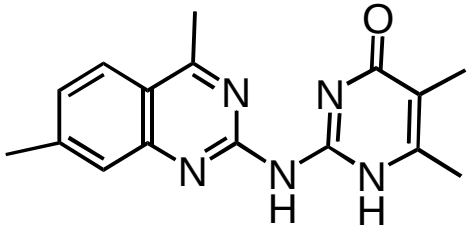
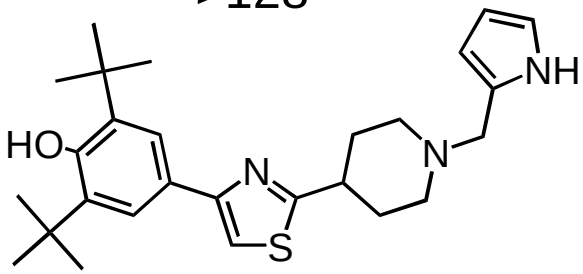
In vitro Activities of Two *S. aureus* Replicase Inhibitors

	Compound 1	Compound 2
IC_{50} (µM), *S. aureus* replicase	18 ± 7.1	7.9 ± 0.2
IC_{50} (µM), mammalian DNA replicase	>50	>50
IC_{50} (µM), DNA binding assay	>50	>50
IC_{50} (µM), HeLa cytotoxicity assay	>100	14
MIC (µg/mL)		
• *S. aureus* ATCC 13709 (MSSA)	4	8
• *S. aureus* ATCC 13709 + 4% HSA	64	64
• *M. bovis* BCG (Denmark, Phipps)	4	n.d.
• *H. influenzae* ATCC 49766	>32	>128
Structure		

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Summary

- **ORF168 and ORF240 polypeptides:**
  - inhibit DNA synthesis selectively in *S. aureus*
  - bind selectively to the *S. aureus* DNA sliding clamp *in vitro*
  - inhibit processive DNA replication *in vitro*

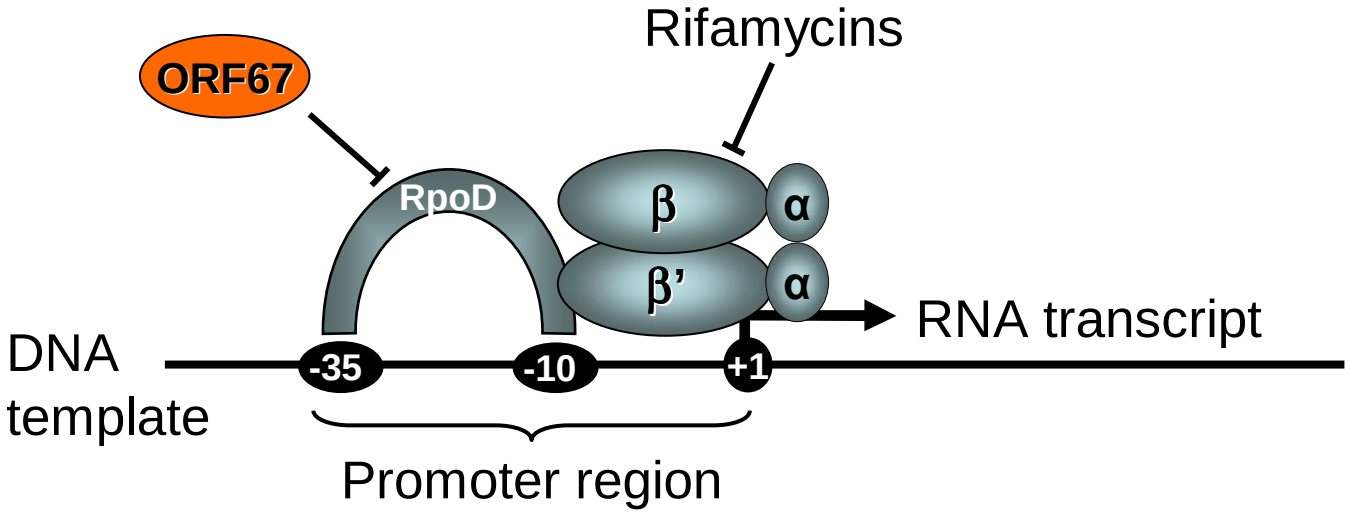
- **Small-molecule inhibitors from replicase screen**:
  - active against G+ including MRSA, and efflux-deficient G-
  - validate the replicase for further inhibitor screening
  - suffer from serum shift
  - lack efficacy in rigorous models of *S. aureus* infection

- **Additional series are under study**

S. aureus transcription target: primary sigma factor RpoD

S. aureus RpoD is the target of phage polypeptide ORF67

- RpoD:
  - primary sigma factor in *S. aureus* transcription machinery
  - ortholog of *E. coli* σ^{70}
  - essential for *S. aureus* viability
  - is targeted by phage polypeptide ORF67



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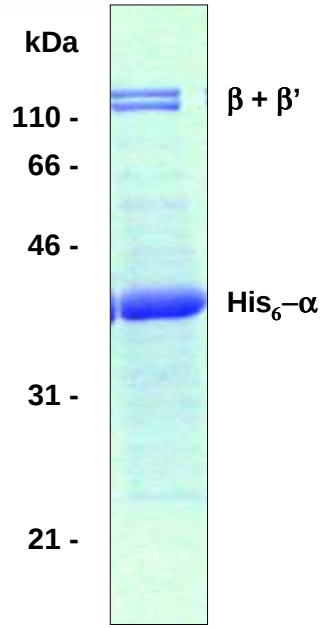
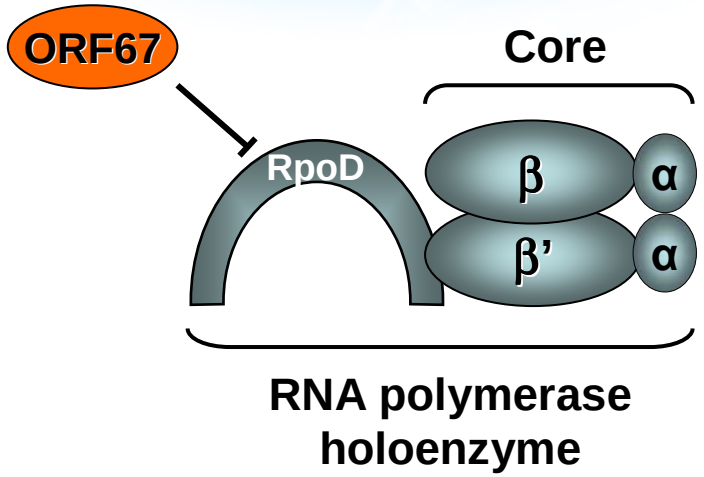
Purification of RNAP Core From *S. aureus*

• Purification of RNAP from *S. aureus* using His-tagged α subunit→ develop *in vitro* assay for RNA synthesis:





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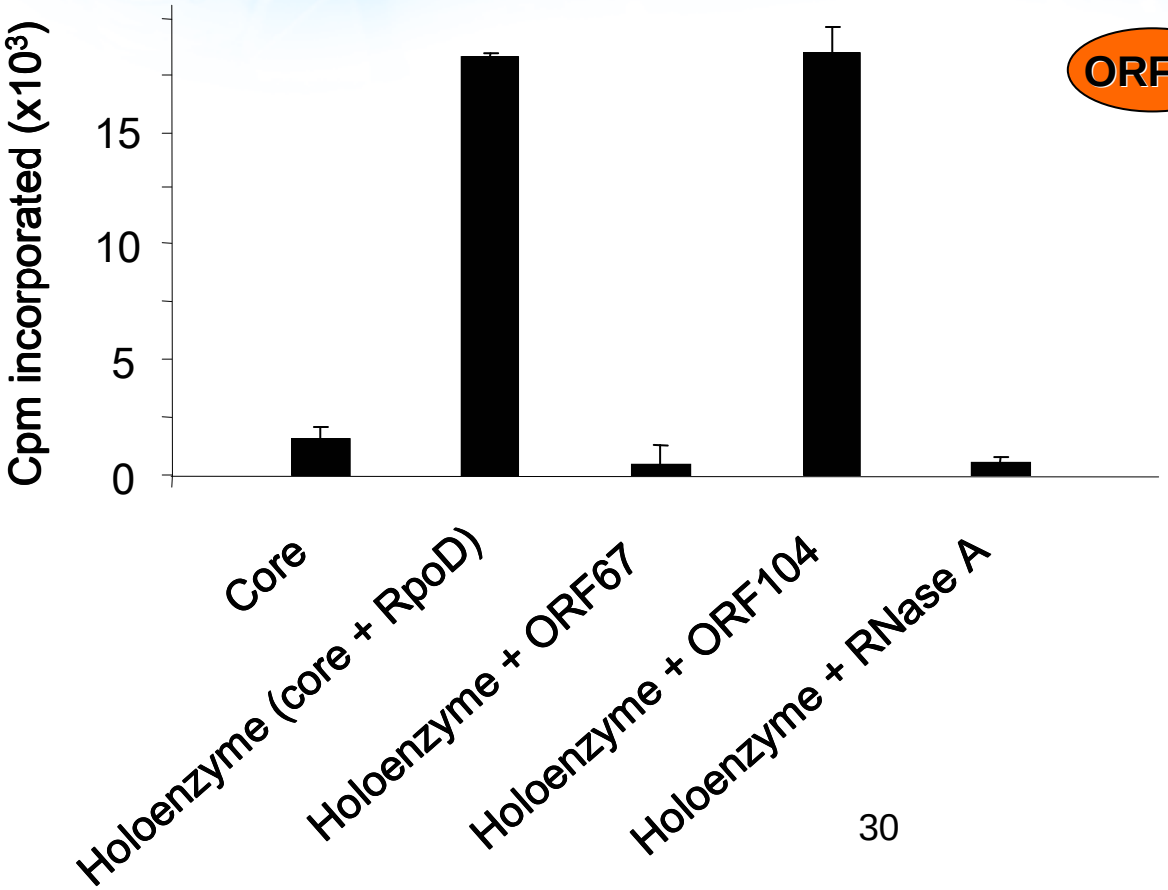
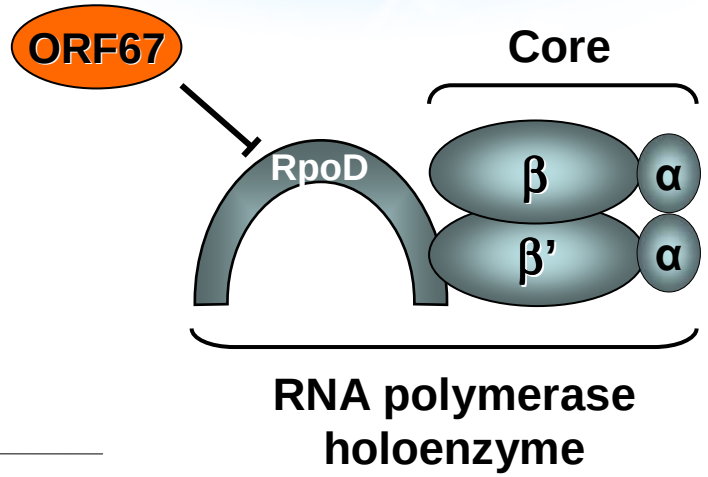
RpoD-Specific Phage Polypeptide (ORF67) Inhibits *S. aureus in vitro* Transcription

• Plate-based assay with purified *S. aureus* RNAP → study effect of phage polypeptides on RNA synthesis *in vitro* and conduct HTS:





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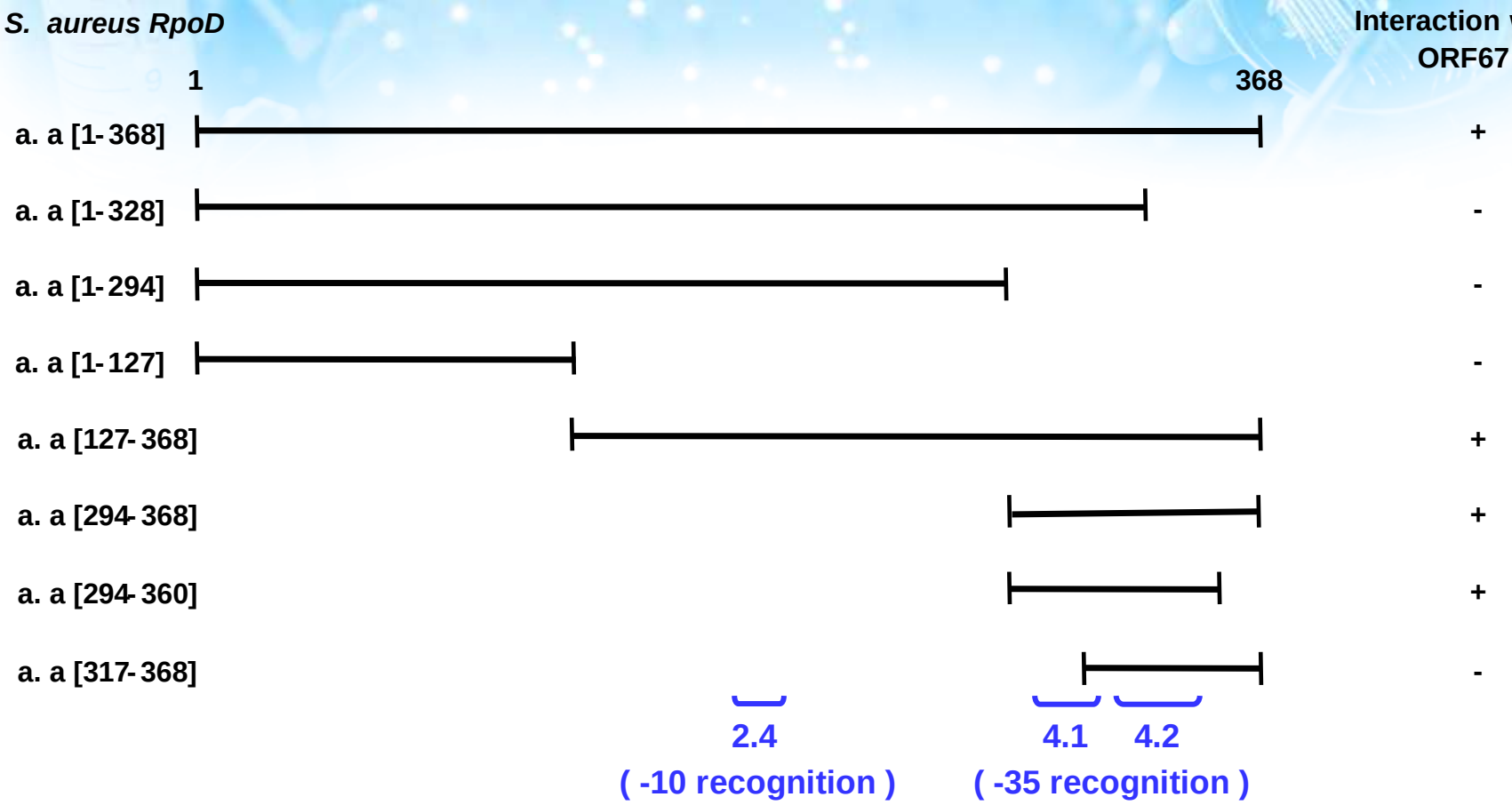
Regions 4.1 and 4.2 of *S. aureus* RpoD Comprise the Minimal Domain For ORF67 Interaction



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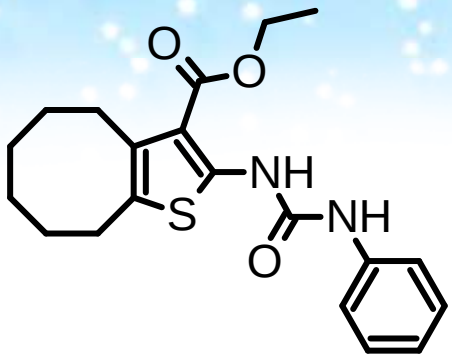
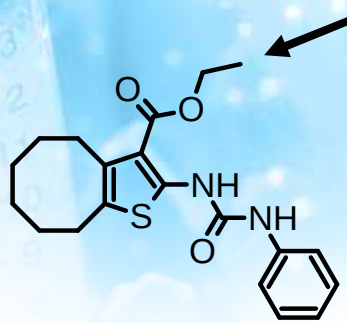
Transcription Screen Identifies a Novel Ureidothiophene Carboxylate Inhibitor



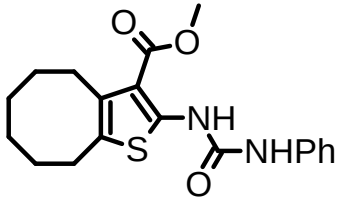
⇒ IC50 (*in vitro S. aureus* transcription):	0.73 µM
⇒ MIC (*S. aureus* Smith ATCC 13709):	1 µg/mL
⇒ MIC (50% serum):	>128 µg/mL
⇒ Spectrum:	limited to Staphylococci
⇒ IC50 (*in vitro* HeLa cytotoxicity):	>100 µM
⇒ IC50 (*E. coli in vitro* transcription):	>100 µM
⇒ IC50 (mammalian *in vitro* transcription):	>100 µM

Ester Variations and Activity

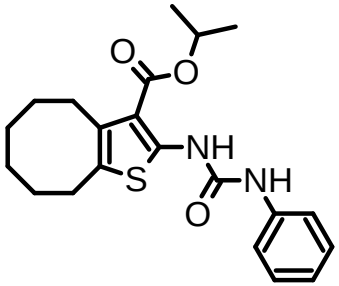


IC50 0.73 µM
MIC 1 µg/mL (Smith)

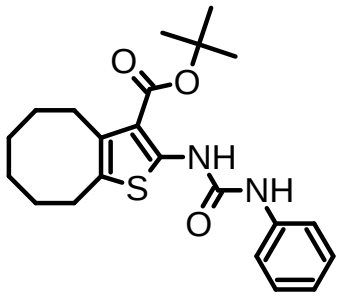
- Ester functionality is necessary
  - amides, ketones, alcohols, acid tested
- Polar groups are undesirable
  - small heterocycles +/- charge tested
- **Isopropyl ester is optimum:**



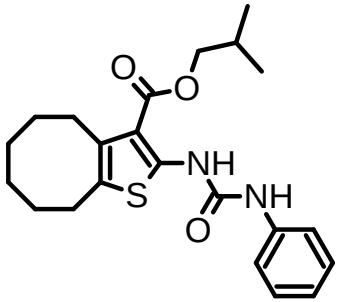
IC50 1.0 µM
MIC >128 µg/mL



IC50 0.06 µM
MIC 1 µg/mL



IC50 0.22 µM
MIC 1 µg/mL



IC50 0.14 µM
MIC >128 µg/mL

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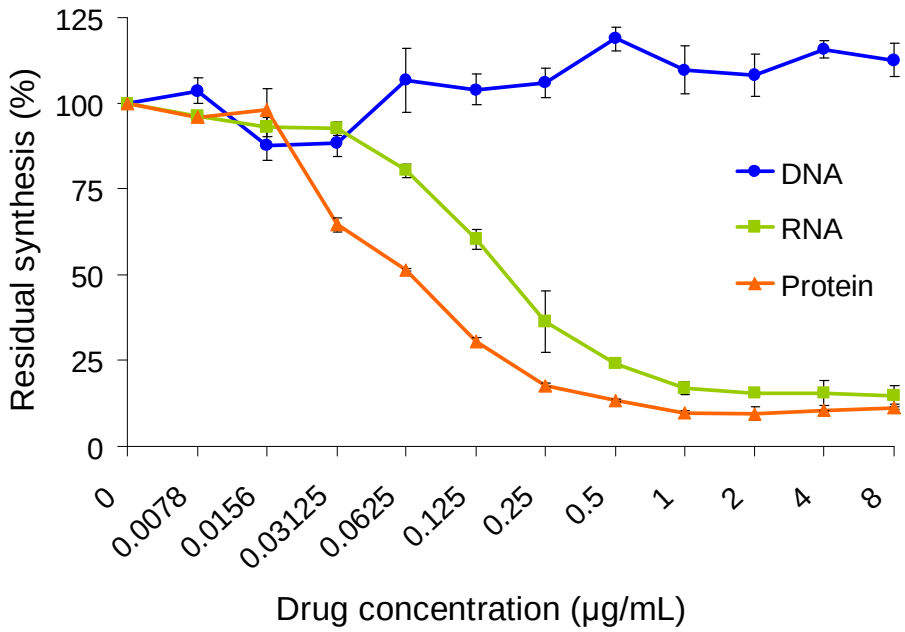
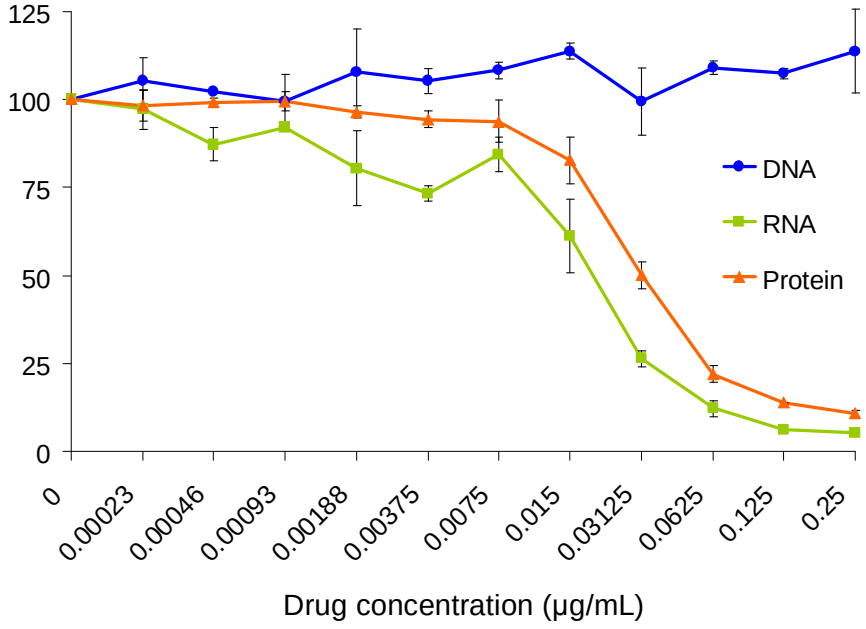
Ureidothiophene Carboxylate Inhibits Transcription in Growing *S. aureus* Cells

• Macromolecular synthesis assay in *S. aureus* → Ureidothiophene carboxylate inhibits RNA and protein synthesis similarly to Rifampicin:

Ureidothiophene carboxylate



Rifampicin



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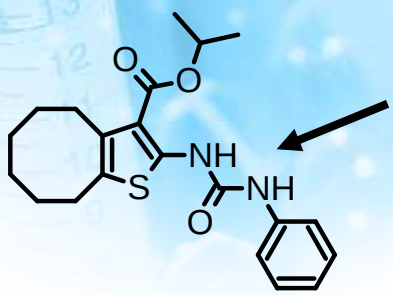
Ureidothiophene Carboxylate is Active Against Antibiotic Resistant Strains of *S. aureus*

Resistant Category	***n***	**MIC or MIC range (μg/mL)**
Mupirocin-resistant	12	0.5 (11 strains) >128 (1 strain)
Rifampicin-resistant	9	< 0.125 - 1
MRSA	14	0.25 - 2
VISA ATCC 700699	1	0.25

- *Activity against Rif^{R} strains suggests distinct binding site or mechanism*

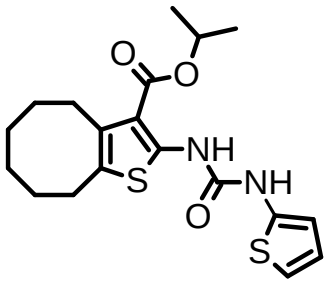
Urea Variations and Activity



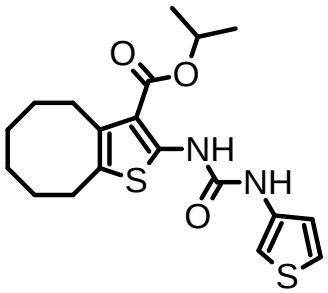
IC50 0.06 µM
MIC 0.5 µg/mL (Smith)

- Urea functionality is necessary; amides, carbamates, thioureas, sulfuric diamides lose inhibitory activity
- Replacement of phenyl ring with alicyclics or heterocyclics abolishes antibacterial activity
- Substituents on phenyl group abolish antibacterial activity
  - meta, para substituents retain inhibitory activity
  - ortho substituents destroy inhibitory activity

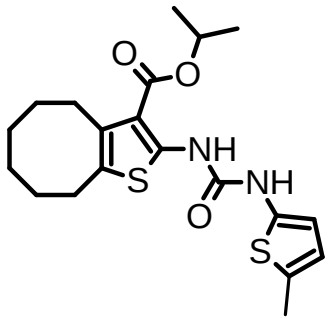
➢ Only thiophenes are tolerated as phenyl replacements:



IC50 0.06 µM
MIC 0.5 µg/mL

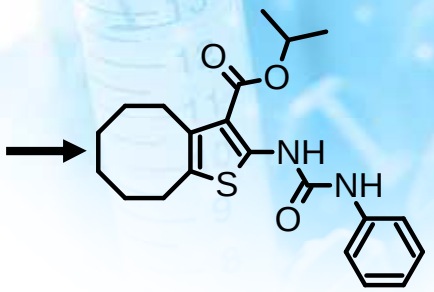


IC50 0.20 µM
MIC 0.5 µg/mL



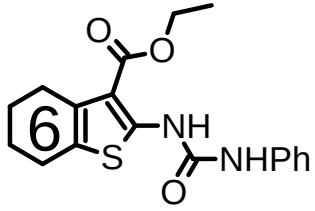
IC50 0.49 µM
MIC 1 µg/mL

Ring Variations and Activity

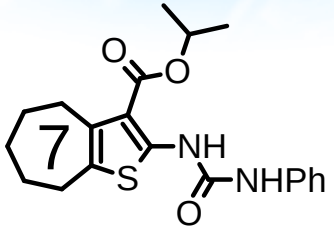


IC50 0.06 µM
MIC 0.5 µg/mL (Smith)

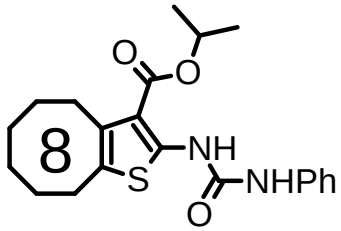
- Heteroatoms in ring abolish antibacterial activity
- Acyclic replacements are detrimental (IC50 5-10 µM)
- **Eight and nine membered rings optimum:**



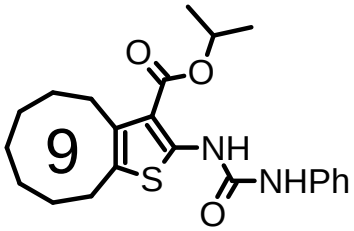
IC50: 2.4 µM
MIC >128 µg/mL



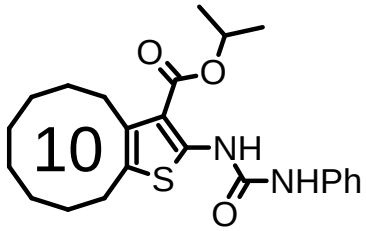
IC50: 0.1 µM
MIC 1 µg/ml



IC50: 0.06 µM
MIC 0.5 µg/mL



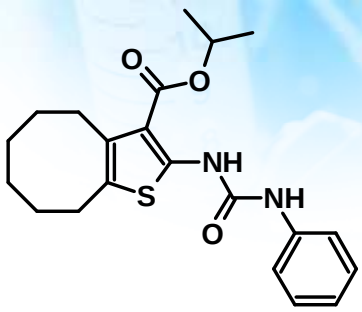
IC50: 0.05 µM
MIC 0.5 µg/mL



IC50: 0.14 µM
MIC >128 µg/mL

Targanta Therapeutics

Ureidothiophene Carboxylate - Summary



IC50 0.06 µM
MIC 0.5 µg/mL

- >120 compounds made in 6 month campaign
- Compound is likely only active against staphylococci and is subject to high frequencies of resistance
- Well-tolerated in mice at 2x25 mg/kg i.v. bolus
- Active in a low-stringency mouse model of systemic *S. aureus* infection (i.p. infection / i.p. injection)
- Additional SAR of hydrophobic ring required to address serum binding issue

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