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Bacteriophage Proteins G1ORF240 and TwortORF168 Validate the DNA Sliding Clamp of *Staphylococcus aureus* for Inhibitor Screening

Adam Belley[1], Mario Callejo[1], Francis Arhin[1], Mohammed Dehbi[1§], Ibtihal Fadhil[1], Jothi Latha Krishnamoorthy[1], Jing Liu[1], Ingrid Sarmiento[1], Ramakrishnan Srikumar[1¶], Pascale Bauda[1], Nhuan Ha[1], Michael Dubow[2], Philippe Gros[3], Jerry Pelletier[3] and Greg Moeck[1].

[1] Targanta Therapeutics, 7170 Frederick Banting, Saint Laurent, QC H4S 2A1 Canada
[2] Université Paris Sud, Orsay, France
[3] McGill University, Montreal, QC, Canada

§ Present address: King Faisal Hospital, Riyadh, Saudi Arabia
¶ Present address: Allergan, Irvine CA

Contact Information
Greg Moeck, PhD
Targanta Therapeutics Inc.
(514) 332-1008 x 232
gmoeck@targanta.com

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ABSTRACT

Background: The *S. aureus* DNA replicase is a complex protein machine comprising a DNA sliding clamp (or β), an ATP-dependent clamp-loader complex and the polymerase PolC. β tethers PolC to DNA and confers upon it remarkable processivity. We demonstrate that the phage proteins G1ORF240 and TwortORF168 exert their bactericidal effect when expressed in *S. aureus* by binding to β and inhibiting DNA synthesis. Based on this validation, we sought a new class of synthetic compounds that could inhibit the growth of *S. aureus*. **Methods:** Our phage functional genomics platform was used to identify and validate the DNA sliding clamp β as the target of phage proteins TwortORF168 and G1ORF240. DNA replication assays used replicase proteins (SSB, PolC, β, δ, δ' and τ) from *S. aureus* and *S. pyogenes* and measured [^{3}H]thymidine incorporation into a primed DNA template. MICs were determined following NCCLS guidelines. **Results:** Bacteriophage proteins TwortORF168 and G1ORF240 bound to the β subunit of *S. aureus* and selectively inhibited DNA synthesis in whole cells. Moreover, G1ORF240 inhibited DNA synthesis in the replicase assay of *S. aureus* (IC50 = 58 nM) but not of *S. pyogenes* (IC50 >1000 nM). The action of G1ORF240 and TwortORF168 were multifaceted and inhibited both loading of β onto DNA and binding of PolC. Screening of a 250,000-compound diversity library for small-molecule inhibitors of the *S. aureus* replicase yielded 2 hits with IC50 <20 μM and MICs against *S. aureus* ATCC 13709 ranging from 2 to 16 μg/mL. Furthermore, these compounds did not inhibit mammalian DNA synthesis (IC50 >50 μM). **Conclusion:** The *S. aureus* replicase, validated by our phage functional genomics platform, represents an ideal target for the development of new antimicrobial agents.

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INTRODUCTION

Bacterial DNA replicases function in the replication of chromosomal DNA and consist of a DNA sliding clamp (β), a clamp-loading complex, and a replicative polymerase. β greatly increases the processivity and incorporation rate of the polymerase during DNA synthesis by tethering it to DNA. Loading of β onto single-stranded DNA is carried out by the ATP-dependent clamp-loader complex composed of δ, δ' and τ subunits. The δ subunit functions to open β and position it around DNA at primed sites of single-stranded DNA. The presence of DNA stimulates the ATPase activity of τ causing the release of β from the clamp loader. Loaded β closes tightly to form a circle around the DNA molecule and allows the replicative polymerase to bind and perform rapid, processive replication of the chromosome.

Bacteriophages, viruses that infect eubacteria, have evolved highly efficient mechanisms to subvert bacterial metabolism and redirect it to phage reproduction. Through intensive analysis of bacteriophage genomes, we identified a number of phage open-reading frames (ORFs) that target proteins of the *Staphylococcus aureus* DNA replication machinery. Two of these phage polypeptides, TwortORF168 and G1ORF240, bind the DNA sliding clamp and are bactericidal when expressed within *S. aureus* cells. Based on the phage validation of this target, we developed an assay that could identify small molecule inhibitors of β-dependent DNA replication *in vitro* and initiated antibacterial drug discovery against *S. aureus*, a human pathogen that causes serious nosocomial infections.

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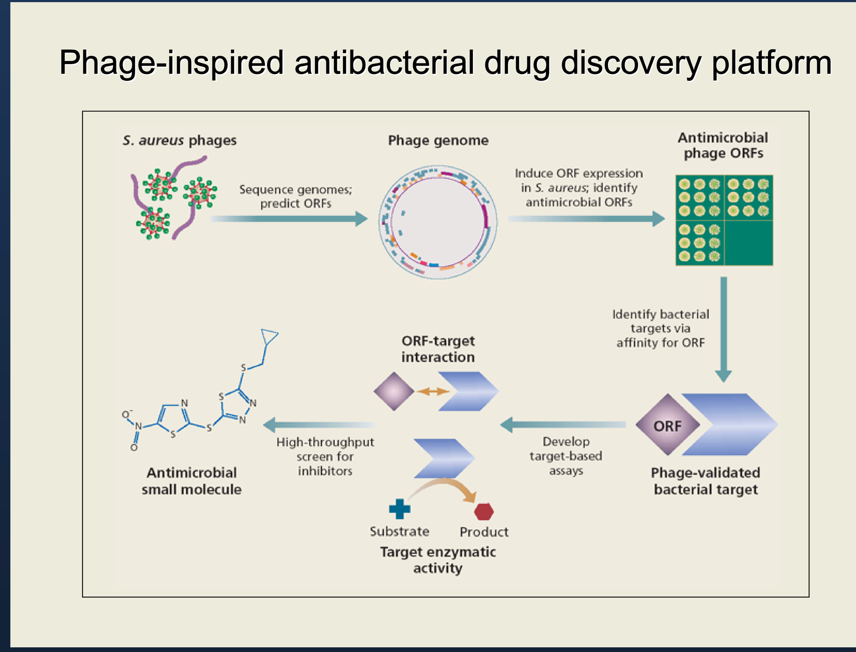
Phage-inspired antibacterial drug discovery platform



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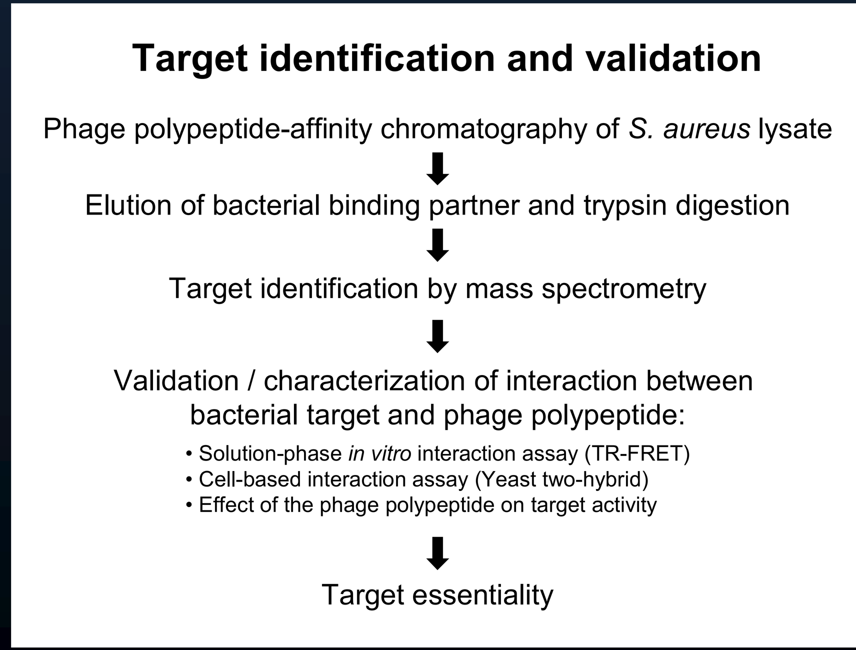
Target identification and validation



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Phage-validated targets

ORF	Bacterial target	Function of target	Essentiality of target
77ORF104 phiPVLORF016	DnaI	Helicase loader	Essential
96ORF78	DnaG	DNA primase	Essential
TwortORF168 G1ORF240	**DnaN (β)**	**DNA sliding clamp**	**Essential**

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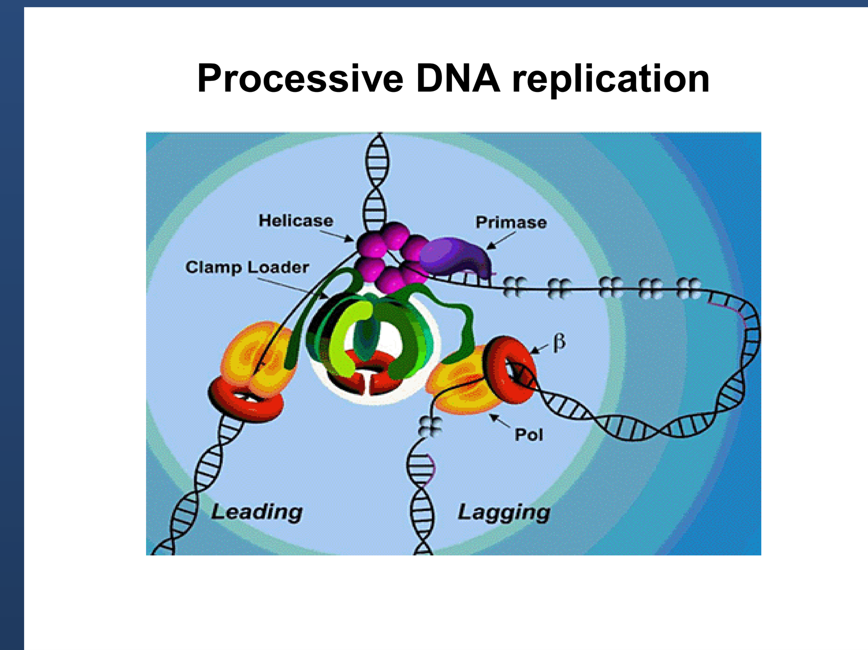
Processive DNA replication



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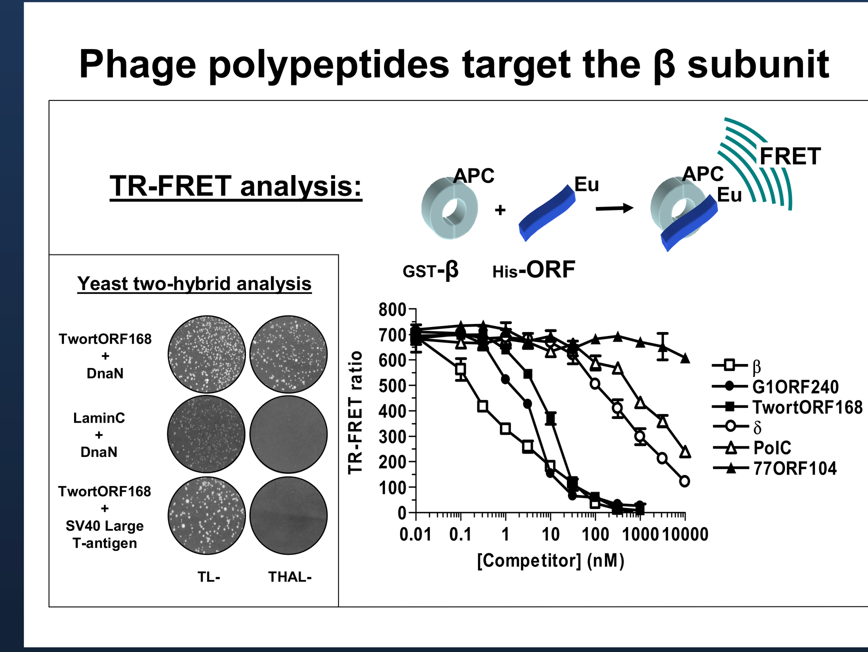
Phage polypeptides target the β subunit



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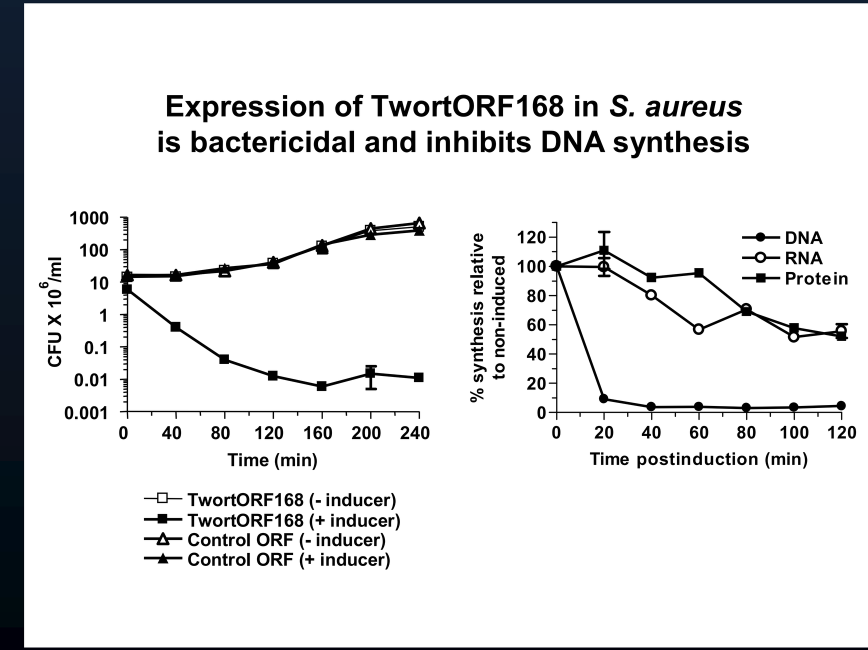
Expression of TwortORF168 in *S. aureus* is bactericidal and inhibits DNA synthesis



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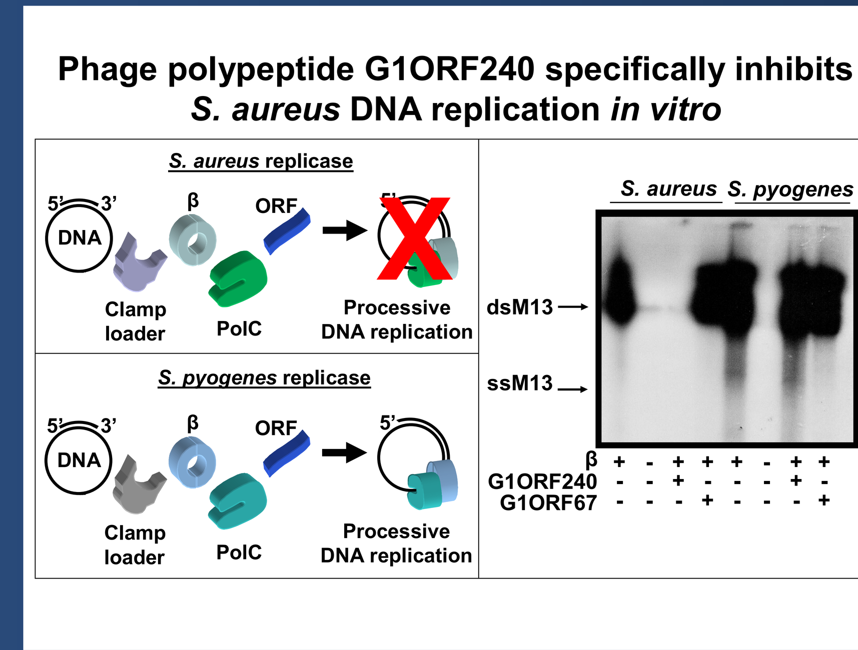
Phage polypeptide G1ORF240 specifically inhibits *S. aureus* DNA replication *in vitro*



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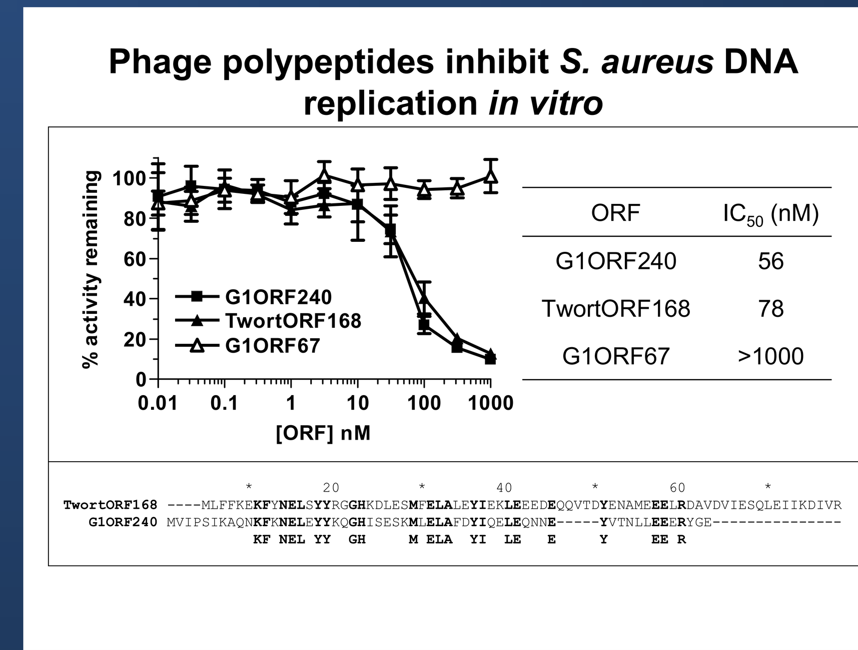
Phage polypeptides inhibit *S. aureus* DNA replication *in vitro*



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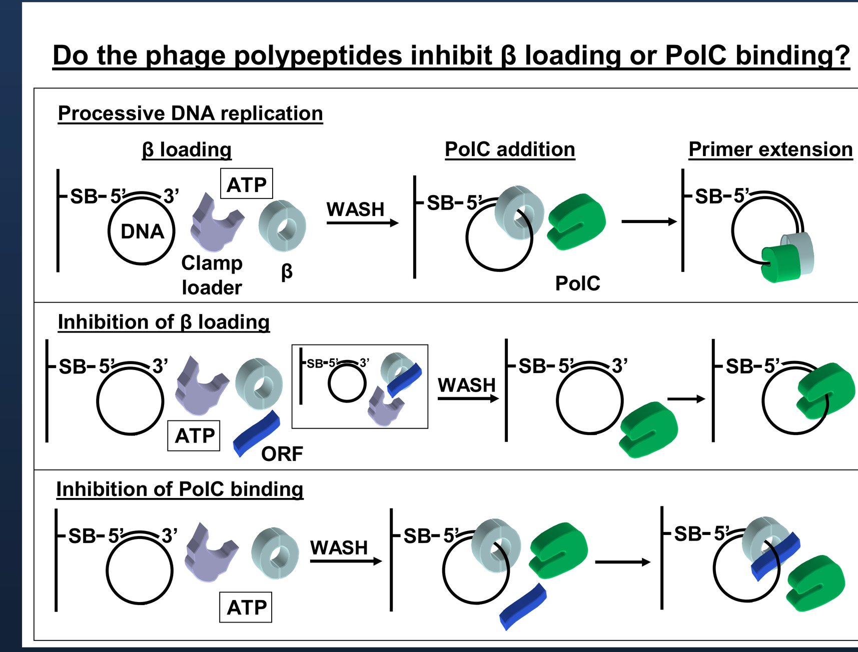
Do the phage polypeptides inhibit β loading or PolC binding?



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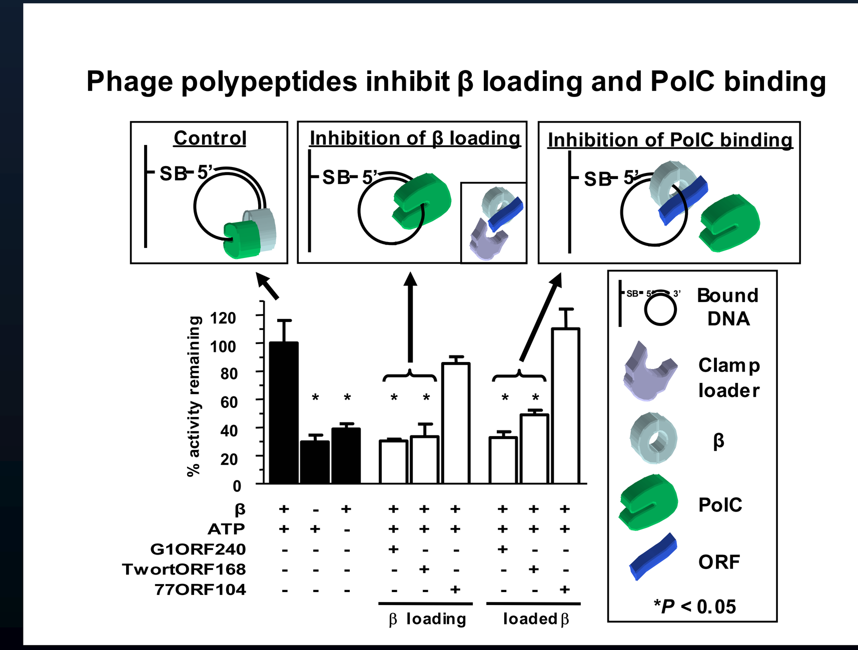
Phage polypeptides inhibit β loading and PolC binding



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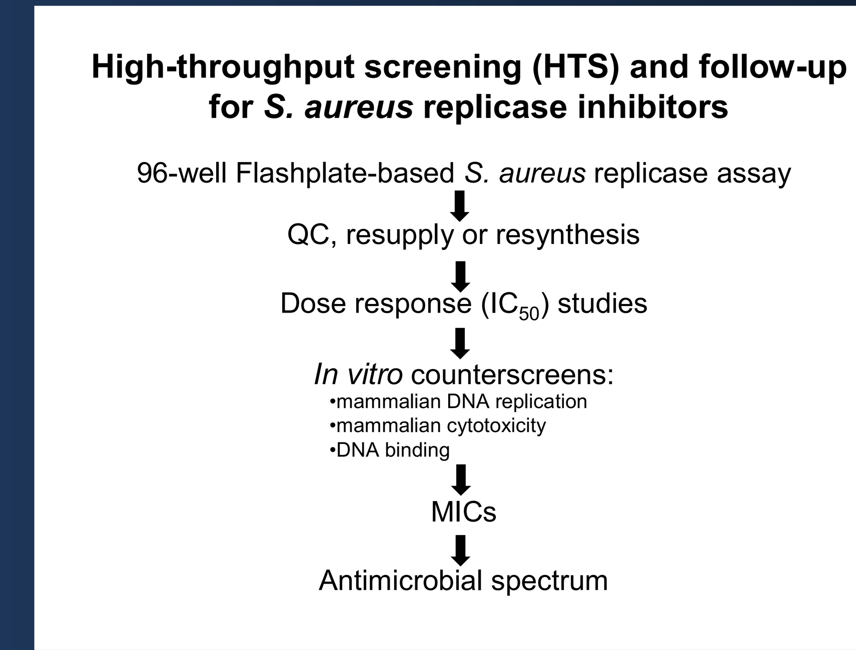
High-throughput screening (HTS) and follow-up for *S. aureus* replicase inhibitors



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Summary of *S. aureus* replicase HTS

Criterion	Number of compounds
Tested in primary HTS assay (20 μM screening dose; n=1)	239,080
Active (singles + pools) in primary HTS assay (40% threshold)	1060
Tested in confirmatory assay (20 μM screening dose; n=3)	2664
Active in confirmatory assay (40% threshold)	126
Tested in dose-response assay (0.32 -100 μM; n=3)	118
Active in dose-response assay ($IC_{50} \leqslant$ 50 μM)	80

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Summary of *in vitro* activities of two *S. aureus* replicase inhibitors

	Compound 1	Compound 2
IC_{50} (μM) in *S. aureus* replicase FlashPlate assay	18 ± 7.1	7.9 ± 0.2
IC_{50} (μM) in mammalian DNA replication assay	>50	>50
IC_{50} (μM) in mammalian cytotoxicity assay	>100	13.5
IC_{50} (μM) in DNA binding assay	>50	>50
MIC (μg/ml)		
• *S. aureus* RN4220	4	8
• *S. aureus* ATCC 13709	4	8
• *H. influenzae* ATCC 49766	>32	>128
Structure		

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Summary

- Expression of TwortORF168 in *S. aureus* inhibits DNA synthesis and consequently results in bactericidal activity
- TwortORF168 and G1ORF240 bind specifically to the DNA sliding clamp of *S. aureus* and inhibit its ability to confer processivity to PolC during DNA replication *in vitro*
- The mechanism of inhibition is multifaceted: the phage polypeptides inhibit loading of the DNA sliding clamp onto DNA and prevent its interaction with PolC
- A *S. aureus* replicase assay was developed to screen for small-molecule inhibitors of DNA replication: 2 inhibitors were identified that had antibacterial activity against *S. aureus*
- The phage-validated *in vitro* replicase assay presents a promising approach for discovery of novel antibacterial agents to counter *S. aureus* infections

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Materials and Methods

Antibacterial activity of the phage polypeptide: TwortORF168, encoded by Staphylococcal phage Twort, was cloned in an expression vector under the control of an arsenite-inducible promoter. Time-kill assays were used to monitor the cell density (expressed as CFU/mL) of *S. aureus* RN4220 transformants +/- arsenite.

Target identification and validation: G1ORF240 affinity chromatography was used to identify binding partners in cell lysate prepared from *S. aureus* RN4220. Bound proteins were eluted with 1% SDS and resolved by SDS-PAGE. Proteins binding specifically to G1ORF240 were excised from the gel and identified by mass spectrometry of tryptic digests. Interaction between G1ORF240 and the bacterial target (β) was confirmed by solution-based time-resolved fluorescence resonance energy transfer (TR-FRET) assays and in the yeast two-hybrid system.

Cell-based macromolecular synthesis: Macromolecule synthesis was monitored in *S. aureus* RN4220 following induction of TwortORF168 by quantifying incorporation of radiolabeled precursors ([^{3}H]thymidine, DNA; [^{3}H]uridine, RNA; [^{35}S]Met, protein) into TCA-precipitable material in 96-well plates.

DNA replication: Bacterial DNA replication assays used replicase proteins (SSB, PolC, β, δ, δ' and τ) from *S. aureus* and *S. pyogenes* and measured [^{32}P]dCTP or [^{3}H]thymidine incorporation into a primed DNA template. Reactions were quenched and either resolved by SDS-PAGE or quantified using streptavidin coated Flashplates. The effect of small-molecule inhibitors on mammalian DNA replication *in vitro* was determined using an SV40 DNA replication assay kit from CHIMERx.

Antimicrobial activity testing: The antibacterial activities of small-molecule inhibitors were assessed in liquid media by monitoring the minimum inhibitory concentration (MIC) following NCCLS guidelines for broth microdilution.