F-1845

The Primary Sigma Factor of *Staphylococcus aureus*: From Target Identification to Isolation of Inhibitors of the RNA Polymerase Holoenzyme

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Abstract

Background: Transcription is essential for cell viability and the transcription machinery presents ideal targets for drug discovery. In bacterial transcription, promoter specificity is governed by one of several sigma factors that associate with the RNA polymerase core enzyme. We discovered and characterized a sigma-binding phage polypeptide and established a screen for inhibitors of sigma-dependent transcription.

Methods: Target identification/validation was performed using a phage functional genomics platform[1] interrogating a growth inhibitory polypeptide of staphylococcal phage G1 (G1ORF67)[2] and crude *S. aureus* lysate. *In vitro* transcription assays monitored *S. aureus* RNA polymerase holoenzyme-dependent ^{32}P-UTP incorporation into acid-precipitable product. MICs were determined by CLSI broth microdilution.

Results: We identified the primary sigma factor of *S. aureus* (σ^{SA}) as the direct target of G1ORF67. In functional assays, G1ORF67 inhibited σ^{SA}-dependent *in vitro* transcription. We screened 250,000 member synthetic diversity library for small molecule inhibitors of *S. aureus* holoenzyme and identified several classes of compounds. The good potency and the lack of cytotoxicity of some inhibitors prompted us to investigate their antibacterial activity. One molecule had an IC_{50} of 1 µM in the *in vitro* transcription assay and MICs of 0.25-2 µg/mL against *S. aureus* strain ATCC 13709 and *S. epidermidis* ATCC 12228.

Conclusion: *S. aureus* σ^{SA} was identified as a target of G1ORF67 and its activity is abolished by the phage polypeptide. Potent and selective inhibitors of *S. aureus* RNA polymerase, with antibacterial activity, were identified from the high throughput screen.

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Introduction

Bacterial RNA polymerase (RNAP) is a clinically-validated target as witnessed with the successful use of Rifampicin over the last four decades. However, the efficacy and low cost of Rifampicin are undermined by the high frequency of resistance to this antibiotic. Furthermore, point mutations in *rpoB* (encoding the β subunit of RNAP) that confer high-level Rifampicin resistance also confer resistance to the other Rifamycins.

To identify novel targets for antibacterial drug discovery, we developed a phage genomics / functional genomics platform (Fig. 1). With this platform, we previously identified several essential bacterial proteins that regulate key metabolic pathways such as DNA replication[1]. Here, we characterized an antibacterial polypeptide termed G1ORF67 from Staphylococcal phage G1[2] and demonstrated that it binds to the primary sigma factor (σ^{SA}) of *S. aureus.* Association of one of several sigma factors with the RNAP core enzyme ($\alpha_2\beta\beta'$) determines promoter specificity of the holoenzyme; in *S. aureus*, σ^{SA} is essential for the expression of housekeeping genes. Phage-based validation has therefore yielded a new target within the RNAP for antimicrobial drug discovery.

In the present study, we characterized the functional consequences of the interaction between G1ORF67 and σ^{SA}. Screening for small molecule inhibitors of *S. aureus* RNAP led us to several classes of compounds with antibacterial activity against *S. aureus*. One particular class, a 2-ureidothiophene-3-carboxylate ester, was selected for determination of structure-activity relationships through a medicinal chemistry program to improve its potency against RNAP *in vitro* and its antibacterial activity.

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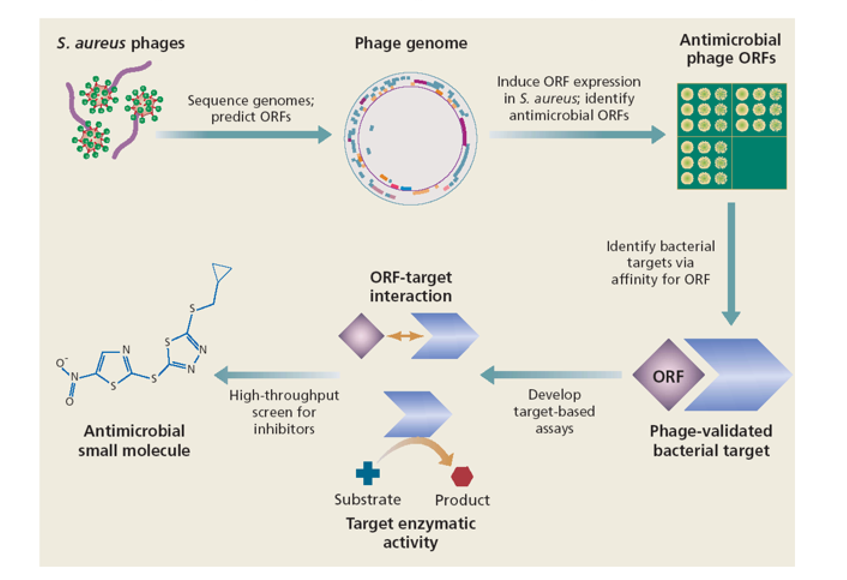
Phage-inspired Drug Discovery Platform



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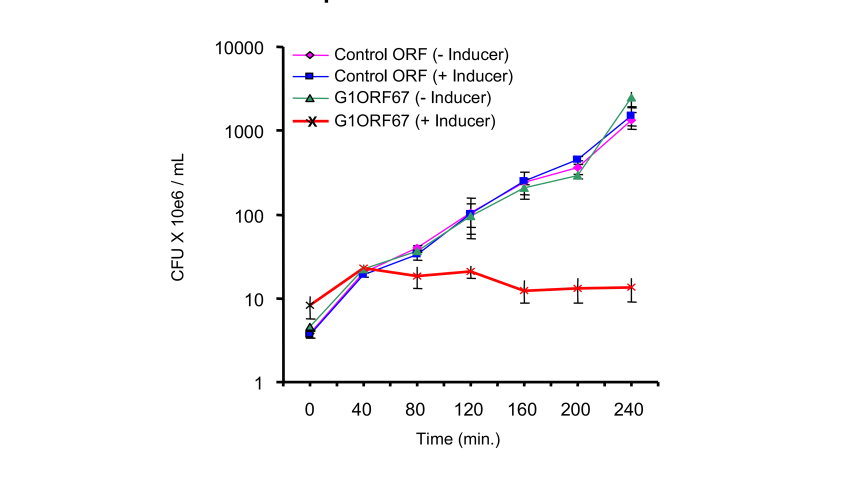
G1ORF67 Exerts a Bacteriostatic Effect When Expressed in *S. aureus*



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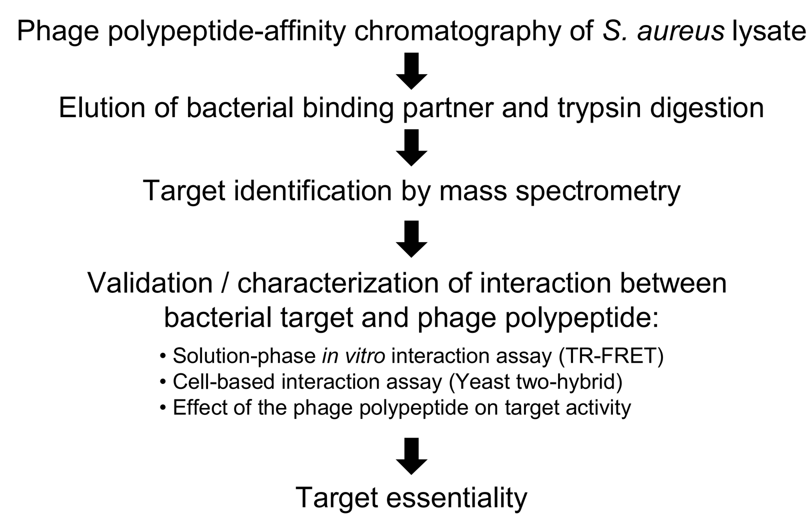
Target Identification and Validation



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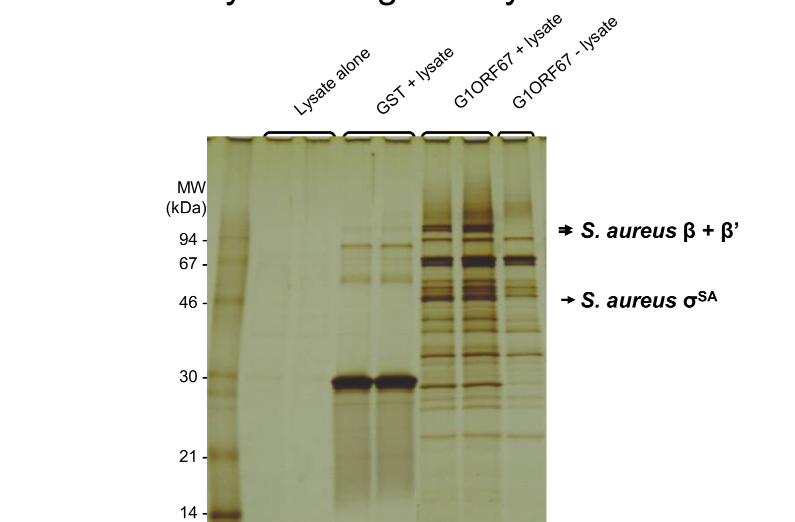
Components of the *S. aureus* Transcription Machinery are Targeted by G1ORF67



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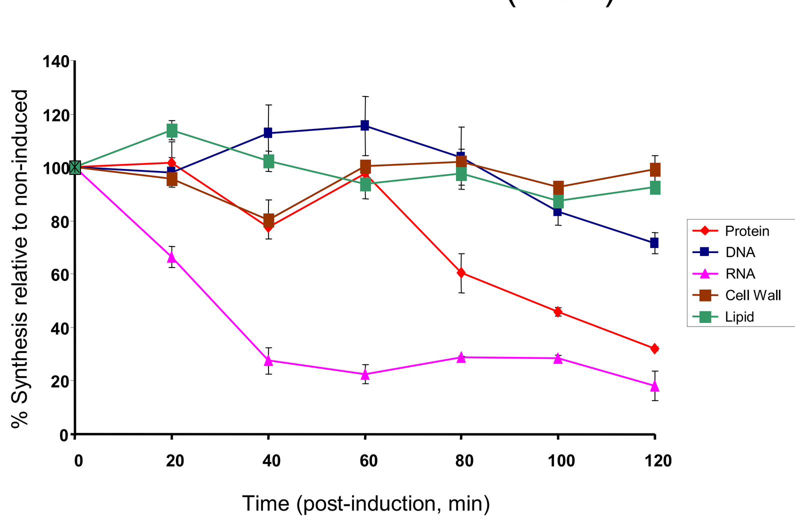
G1ORF67 Specifically Inhibits Transcription in *S. aureus* Mode-of-Action (MOA) Studies



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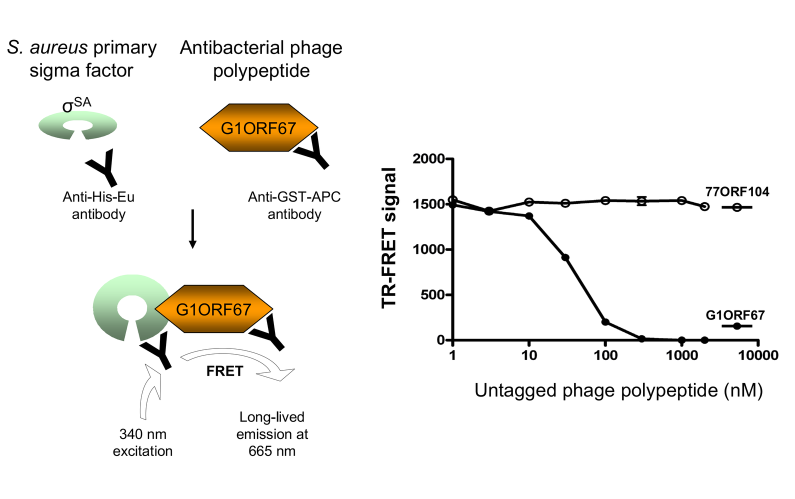
Confirmation of the Direct Interaction Between G1ORF67 and *S. aureus* σ^{SA} by TR-FRET



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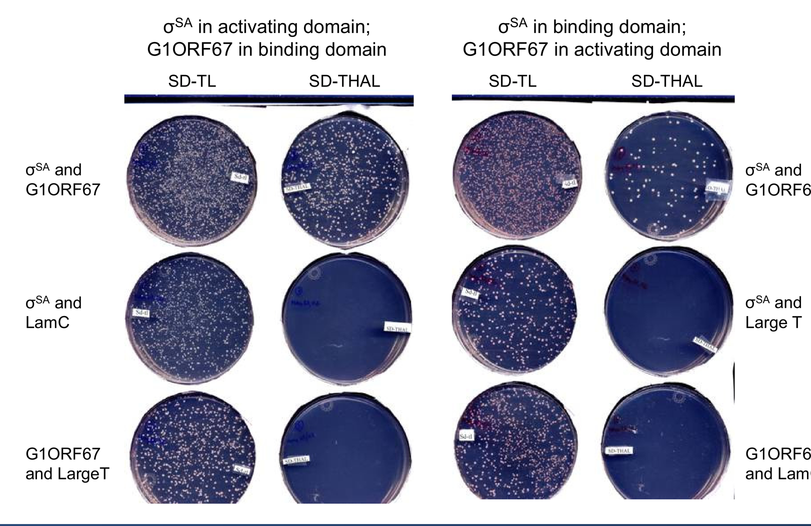
Confirmation of the G1ORF67 - σ^{SA} Interaction in the Yeast Two-hybrid System



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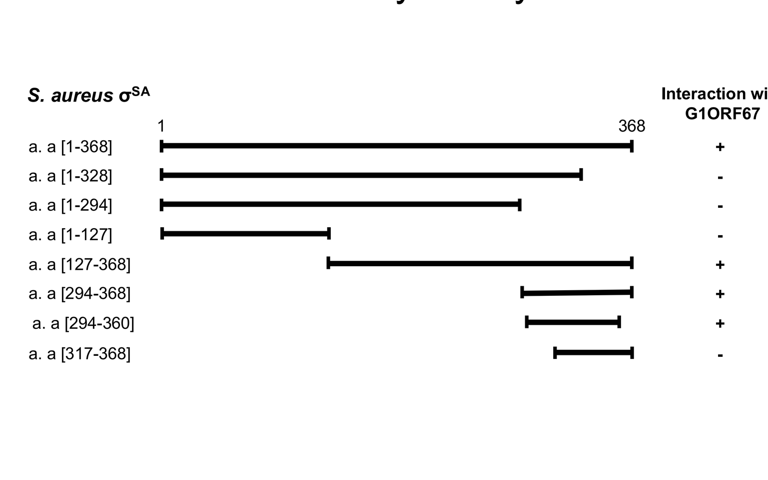
Identification of the Minimal Domain of σ^{SA} Conferring Interaction With G1ORF67 in the Yeast Two-hybrid System



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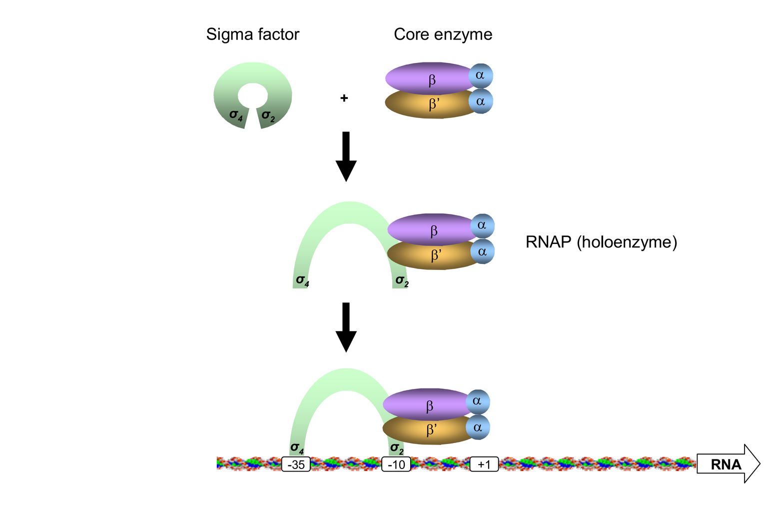
Sigma Factor Confers Promoter Specificity to the RNA Polymerase Core Enzyme



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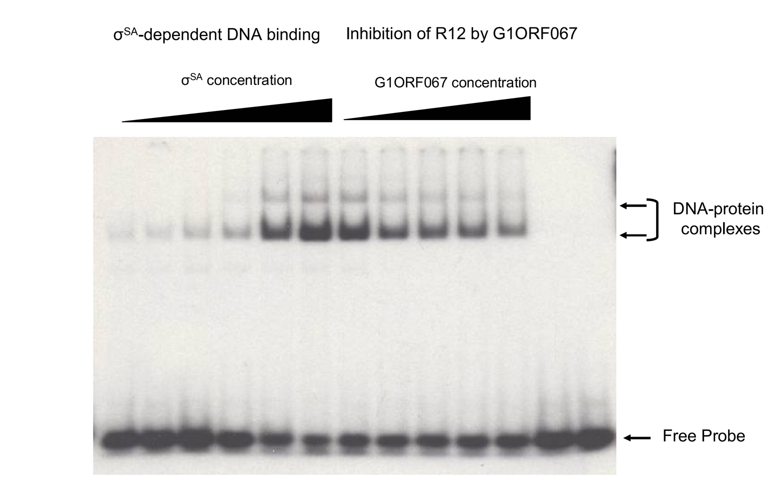
G1ORF67 Inhibits the Binding of σ^{SA} to Promoter DNA in EMSA



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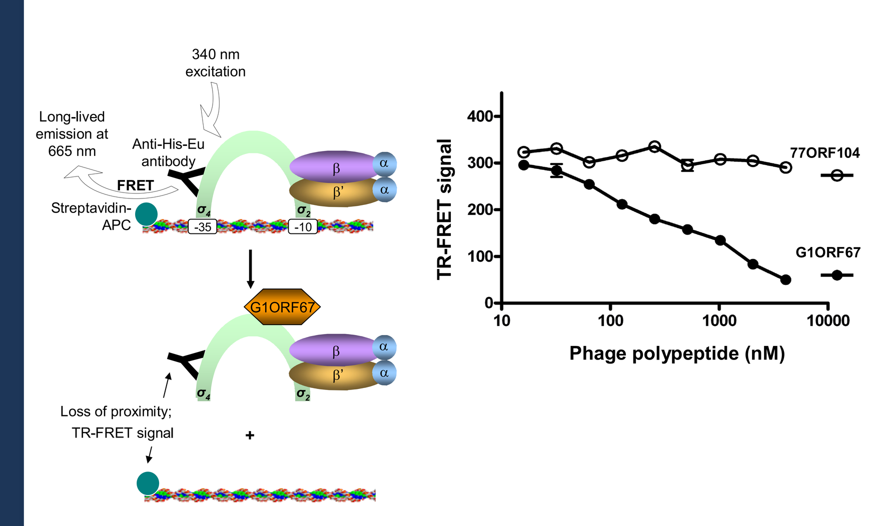
G1ORF67 Inhibits the Binding of σ^{SA} to Promoter DNA in TR-FRET-based DNA Binding Assays



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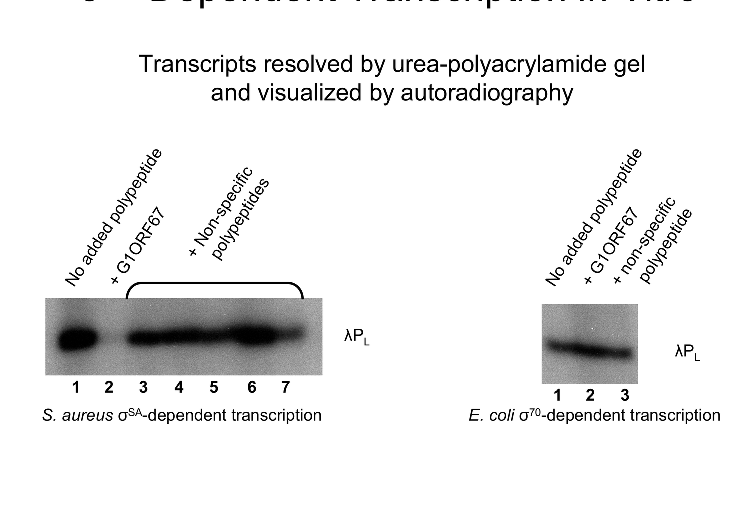
G1ORF67 Specifically Inhibits *S. aureus* σ^{SA}-Dependent Transcription *In Vitro*



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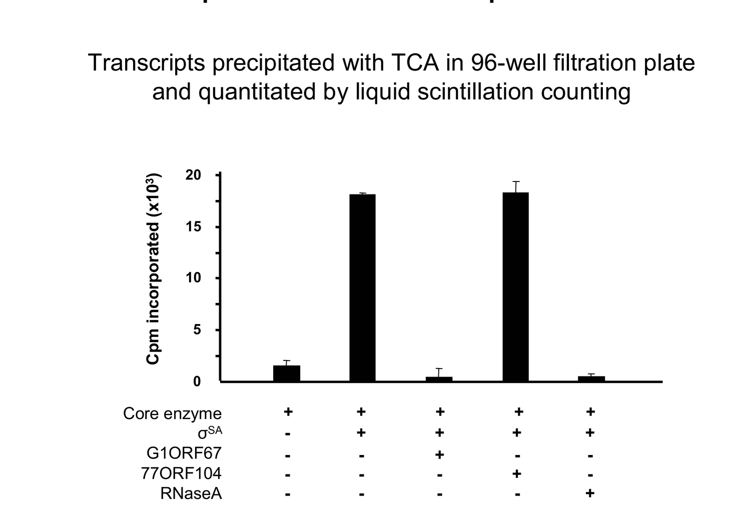
G1ORF67 Specifically Inhibits *S. aureus* σ^{SA}-Dependent Transcription *In Vitro*



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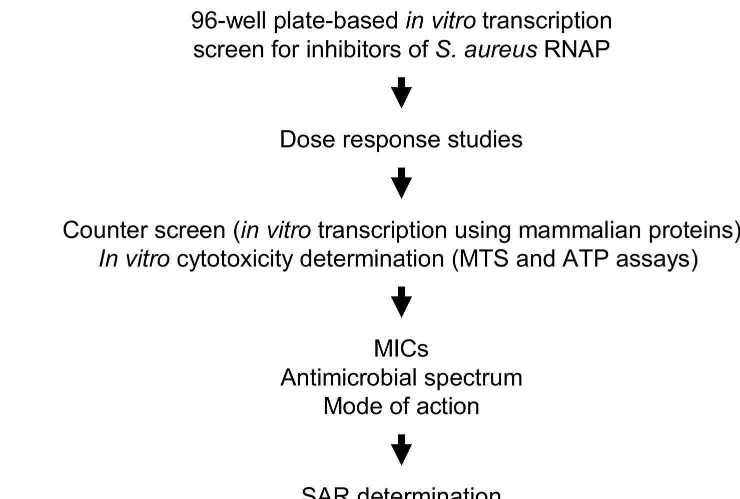
High-Throughput Screening (HTS) and Follow-up for RNAP Inhibitors



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In vitro Potency and Antibacterial Activity of 2-Ureidothiophene-3-Carboxylate Esters

Compound	IC50 (µM), *In vitro* transcription		MIC (µg/ml)			
	S. aureus RNAP	*E. coli* RNAP	*S. aureus* ATCC 13709	*S. aureus* RN4220	*S.epidermidis* ATCC 12228	*E. coli* LBB925 *tol*C
(1)	1	>100	1	>128	0.25	>128
(2[5])	0.06	>100	1	1	0.25	>128

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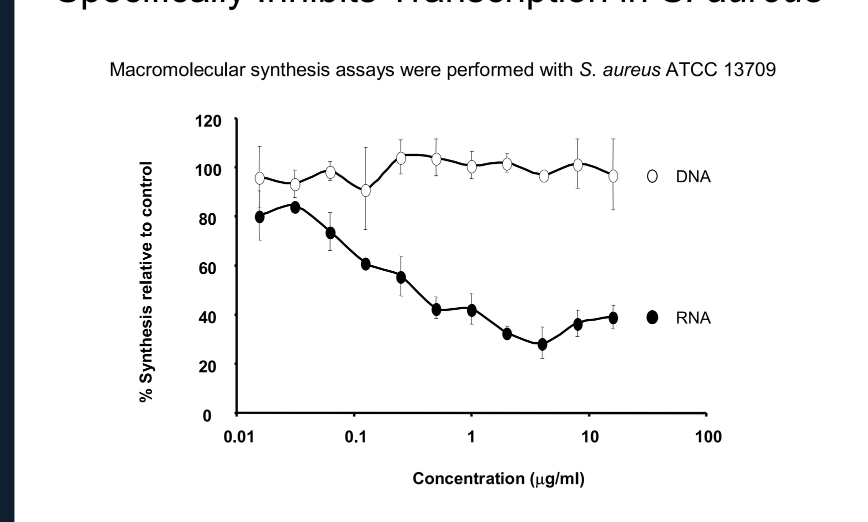
The 2-Ureidothiophene-3-Carboxylate Ester (1) Specifically Inhibits Transcription in *S. aureus*



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Summary

1. Expression of G1ORF67 in *S. aureus* rapidly inhibits transcription- the likely mechanism for host shut-off during phage infection- and prevents cell growth
2. The primary sigma factor of *S. aureus,* σ^{SA}, was identified as the target of the G1ORF67 polypeptide
3. G1ORF67 is a potent inhibitor of σ^{SA}-dependent transcription and DNA binding in *in vitro* functional assays with the reconstituted RNA polymerase holenzyme
4. A σ^{SA}-dependent transcription assay was developed for inhibitor screening and small molecule inhibitors of *S. aureus* RNA polymerase were identified
5. The 2-ureidothiophene-3-carboxylate ester RNAP inhibitors selectively inhibit *S. aureus* transcription *in vitro* and display antibacterial activity against *S. aureus*
6. These inhibitors have been further characterized by an intensive medicinal chemistry study in an effort to determine structure-activity relationships; results of this study are presented in Poster F-1874[5]

References

1- Liu, J. et al. (2004) Nat. Biotechnol. *22*: 185
2- Kwan, T., et al. (2005) PNAS, 102:5174
3- Deora and Misra (1996) J. Biol. Chem. 271:21828
4- Fenton, M. et al. (2000) EMBO J. 19:1137
5- Rafai Far, A. et al. (2005) 45th ICAAC meeting, poster F-1874

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Materials and Methods

Antibacterial activity of the phage polypeptide: G1ORF67, encoded by Staphylococcal phage G1[2], 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: G1ORF67 affinity chromatography[1] 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 G1ORF67 were excised from the gel and identified by mass spectrometry of tryptic digests. Interaction between G1ORF67 and the bacterial target (σ^{SA}) was confirmed by solution-based time-resolved fluorescence resonance energy transfer[1] (TR-FRET) assays and in the yeast two-hybrid system.

In vitro transcription assays: *In vitro* transcription assays[3] used a plasmid template and *S. aureus* σ^{SA} complemented with *S. aureus* or *E. coli* core RNAP enzyme. The ^{32}P-labeled RNA product was analyzed by gel electrophoresis. For high-thoughput screening, the acid-precipitable product was quantitated by scintillation counting in 96-well Millipore MultiScreen GF/B plates.

DNA-binding studies: Electrophoretic mobility shift assays and TR-FRET DNA-binding assays were used to monitor binding of *S. aureus* σ^{SA} to an oligonucleotide derived from λP_R promoter[4].

Antimicrobial activity testing: The antibacterial activity of small molecule compounds and control antibiotics was assessed in liquid media by monitoring the minimum inhibitory concentration (MIC) following CLSI guidelines for broth microdilution.

Cell-based macromolecular synthesis: Macromolecule synthesis was monitored in *S. aureus* 13709, following exposure to compounds or induction of G1ORF67, by quantitating incorporation of radiolabeled precursors (^{3}H-thymidine, DNA; ^{3}H-uridine, RNA; ^{35}S-Met, protein; ^{3}H-glycerol, lipid; ^{3}H-N-acetyl glucosamine, cell wall) into TCA-precipitable material in 96-well plates.