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Antimicrobial drug discovery through bacteriophage genomics

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Over evolutionary time bacteriophages have developed unique proteins that arrest critical cellular processes to commit bacterial host metabolism to phage reproduction. Here, we apply this concept of phage-mediated bacterial growth inhibition to antibiotic discovery. We sequenced 26 *Staphylococcus aureus* phages and identified 31 novel polypeptide families that inhibited growth upon expression in *S. aureus*. The cellular targets for some of these polypeptides were identified and several were shown to be essential components of the host DNA replication and transcription machineries. The interaction between a prototypic pair, ORF104 of phage 77 and DnaI, the putative helicase loader of *S. aureus*, was then used to screen for small molecule inhibitors. Several compounds were subsequently found to inhibit both bacterial growth and DNA synthesis. Our results suggest that mimicking the growth-inhibitory effect of phage polypeptides by a chemical compound, coupled with the plethora of phages on earth, will yield new antibiotics to combat infectious diseases.

The Gram-positive bacterium *S. aureus* is an important cause of severe infections in immunocompromised individuals and is a major source of nosocomial infections in hospitalized patients in North America[1]. The latter problem is compounded by the widespread appearance of drug-resistant clinical isolates, particularly methicillin- and vancomycin-resistant *S. aureus*[2,3]. To meet this challenge, significant efforts have been applied to the development of new and effective antibiotics. A prerequisite for target-based antibiotic drug discovery for bacteria such as *S. aureus* is the identification of proteins that are both essential for growth and susceptible to inhibition by small molecules or natural products. The completion of the sequence of the *S. aureus* genome[4] allows the use of functional genomics strategies to identify essential genes and proteins for new antimicrobial drug development[5,6]. Such strategies include the isolation of temperature-sensitive mutants or systematic gene inactivation by antisense RNA expression or chromosomal gene insertion/deletion[7–9]. Although these approaches have yielded hundreds of candidate protein targets, many proteins may not be accessible to small-molecule inhibitors or may be overly abundant within the cell. In addition, it may be difficult to develop *in vitro* screening assays of compound libraries for them. Thus, new approaches are required to prioritize and select the most promising candidate proteins for drug discovery.

Over the course of evolution, bacteriophages, the viruses of eubacteria, have developed unique proteins that bind to and inactivate (or redirect) critical cellular proteins in bacteria, shutting off key metabolic processes to divert host metabolism to the production of progeny phages. In the well-studied phages of *Escherichia coli*, host physiology shutoff is typically performed early during the phage lytic cycle by small phage-encoded proteins that target particularly vulnerable and accessible proteins involved in crucial host metabolic processes. For example, the product of bacteriophage T7 gene 2 (gp2) binds to *E. coli* RNA polymerase and inhibits transcription[10]. The AsiA protein of phage T4 inhibits the bacterial RNA polymerase σ70 transcription factor, freeing the core polymerase to interact with T4-specific sigma factors[11]. Proteins P of phage λ and B of phage P2 each bind to and redirect the host DnaB helicase to their respective phage origins of replication[12,13]. Thus, information in bacteriophage genomes could be extracted and used to identify particularly susceptible proteins in their bacterial hosts and to prioritize these targets for drug discovery.

Therapeutic use of bacteriophages has undergone a renaissance in the form of application or ingestion of phage particles ('phage therapy') for the prevention and treatment of bacterial diseases[14,15]. In addition, phage host-cell lysis proteins, encoded by holins and amidases and elaborated late in the infection cycle, maintain their potent antibacterial activity when administered from outside the cell[16,17]. In contrast to these approaches, we sought a druglike chemical compound that could reproduce the antibacterial effect of selected phage polypeptides following their induction within their host. In so doing, we targeted a surface of interaction shown by phage polypeptide binding to result in bacterial growth inhibition.

Using a high-throughput bacteriophage genomics strategy, we exploited the concept of phage-mediated inhibition of bacterial growth

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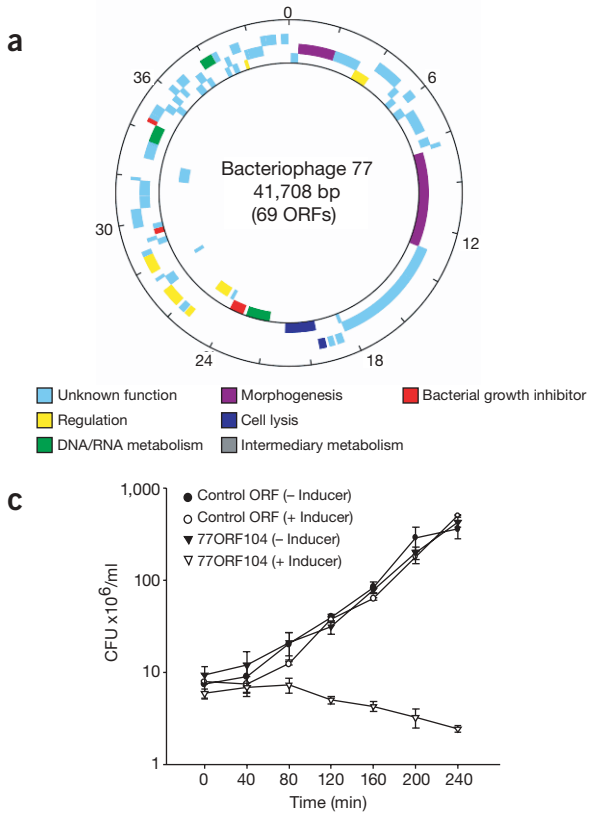


Figure 1 Functional screening for growth-inhibitory polypeptides encoded by phage 77. (**a**) Phage 77 genome map. ORFs were classified into seven functional groups. (**b**) Dot screening. For each ORF, three dilutions of three independent clones of *S. aureus* RN4220 were dotted onto TSA/Kan ± 5 μM $NaAsO_2$. (**c**) Growth inhibition kinetics. Clones of *S. aureus* RN4220 harboring either 77ORF104 or a control noninhibitory ORF were grown in TSB/Kan ± 5 μM $NaAsO_2$. At different time intervals, aliquots of the cultures were plated onto TSA/Kan for determination of colony-forming units. Results are averages of three independent clones for each ORF ± sd. TSA, tryptic soy agar; Kan, kanamycin; TSB, tryptic soy broth; CFU, colony-forming units.

to systematically identify antimicrobial phage-encoded polypeptides. Several cognate protein targets in *S. aureus* were identified, including key components of the DNA replication and RNA transcription machineries. In some cases, sequence-unrelated polypeptides from different phages were found to target the same proteins in *S. aureus*, underscoring the particular susceptibility of these proteins to inhibition and their suitability for antimicrobial drug discovery. Furthermore, small-molecule inhibitors of the interaction between a prototypic cognate pair, ORF104 of phage 77 and DnaI, the putative helicase loader of *S. aureus*, were found to inhibit both bacterial growth and DNA synthesis, validating the premise of mimicking the growth-inhibitory effect of phage polypeptides by a chemical compound. On a fundamental level, the research platform described here can be used to delineate the range of host metabolic pathways that are inactivated by bacteriophages to optimize their propagation. Moreover, in view of the large number of bacteriophages on earth, this approach is being adapted to study other bacterial species to aid in the identification of small molecule leads for drug development and thus to augment the arsenal of antibiotics required to overcome ongoing and future antibiotic resistance.

RESULTS

Characterization and sequencing of *S. aureus* phage genomes

Based on fingerprinting of phage DNA using restriction enzymes and sequence homology as determined by sample sequencing analysis, 150 bacteriophages that had double-stranded DNA genomes and were capable of lytic growth on *S. aureus* were classified into three groups according to their genome size: (i) <20 kilobase pairs (kbp), represented by morphotype C1 phage p68, (ii) ~40 kbp, represented by morphotype B1/B2 phage 77 and (iii) >100 kbp, represented by morphotype A1 phage G1 (ref. 18). A total of 27 of the most distinct phages were selected and the genomes of 26 of them were fully sequenced (the genome sequence of phage phiPVL[19] was available from a public database, GenBank accession no. NC_002321). As an example, the phage 77 genome was found to consist of 41,708 base pairs (bp) and was predicted to contain 69 open reading frames (ORFs) encoding polypeptides of at least 33 amino acids (**Fig. 1a**; GenBank accession no. AY508486).

Functional screening for antimicrobial phage ORFs

To systematically screen phage genomes for gene products inhibiting the growth of *S. aureus*, we cloned predicted phage ORFs under the control of an arsenite-inducible promoter derived from the *S. aureus* chromosomal *ars* operon[20]. The growth of *S. aureus* strain RN4220 (ref. 21) transformants was compared after plating liquid cultures on solid medium in the presence or absence of sodium arsenite. In a representative screening assay, bacterial growth was abolished when the expression of the polypeptide product of ORF104 of phage 77 (77ORF104) was induced (**Fig. 1b**). When we monitored the kinetics of growth inhibition mediated by expression of 77ORF104 within *S. aureus* in liquid culture, the phage polypeptide was found to rapidly exert a bacteriostatic effect, as indicated by an immediate arrest in cell growth (**Fig. 1c**). Immunoblotting of cell lysates from induced cultures revealed that only small quantities of 77ORF104 were expressed (data not shown), suggesting that bacterial growth inhibition was not a result of mass overproduction of 77ORF104. The entire set of 69 predicted ORFs in phage 77 was screened in this manner. Excluding genes encoding the cytolytic polypeptides holin and amidase[22,23], or polypeptides with predicted[24] transmembrane domains (five of the 69 predicted gene products in phage 77), three distinct ORFs, including ORF104, were found to markedly inhibit *S. aureus* growth. We extended this screen to the 895 ORFs from the 26 additional sequenced

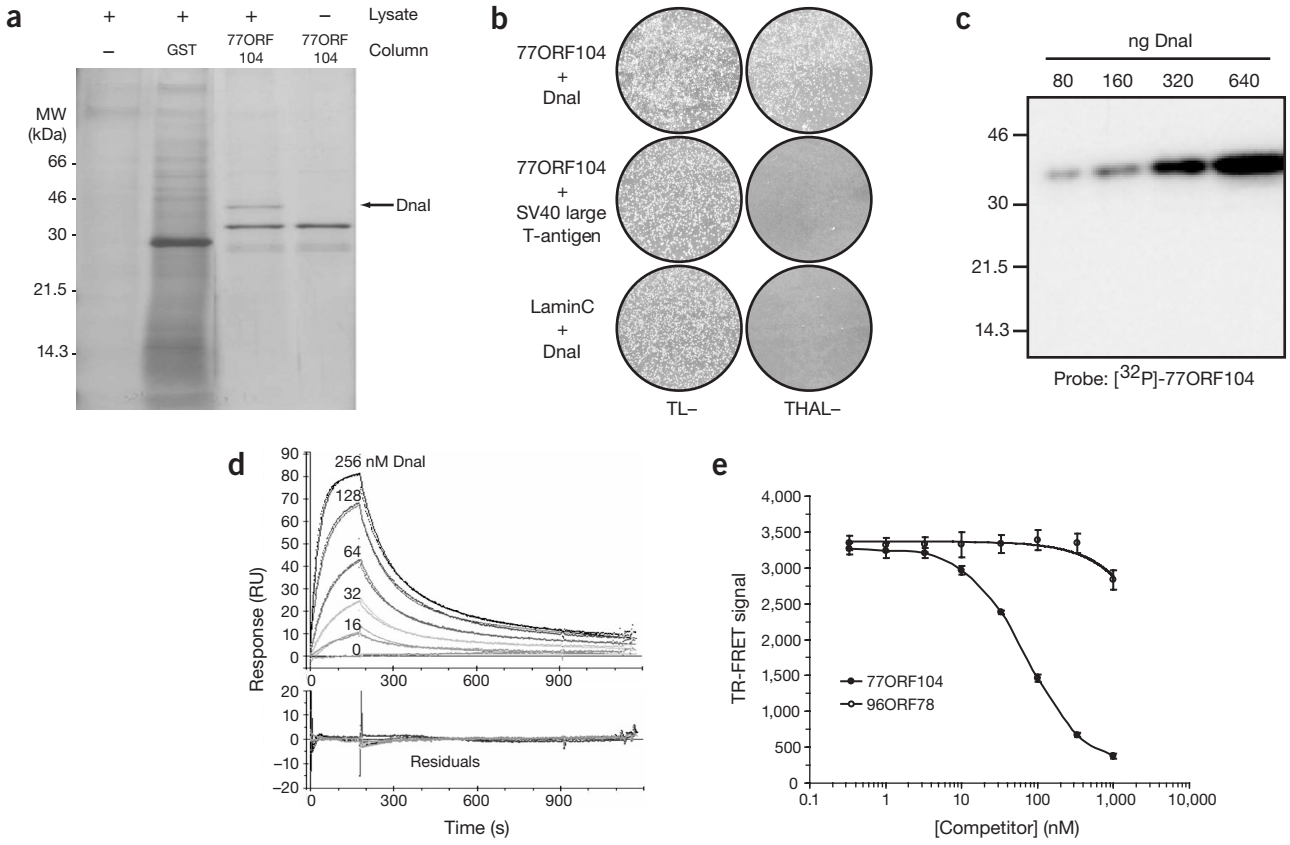


Figure 2 Interaction of 77ORF104 and *S. aureus* DnaI. (**a**) Identification of DnaI by affinity chromatography. The protein band found to interact specifically with 77ORF104 is indicated by an arrow. (**b**) Yeast two-hybrid analysis. 77ORF104 and DnaI cotransformants were plated on synthetic medium lacking tryptophan and leucine (TL–), or lacking tryptophan, histidine, adenine and leucine (THAL–). (**c**) Far-western analysis. Immobilized DnaI was detected with ^{32}P-77ORF104. (**d**) BIAcore analysis. Upper panel, DnaI was injected over a surface of immobilized 77ORF104. After reference subtraction, the data were fitted to a conformational change model (solid lines). Lower panel, residuals obtained after global analysis. RU, resonance units. (**e**) Untagged 77ORF104 competes for binding to DnaI in TR-FRET. The interaction between His-tagged DnaI and GST-tagged 77ORF104 was detected by anti-tag antibodies conjugated to europium and allophycocyanin, respectively. Untagged 77ORF104 was used to compete for DnaI; an untagged ORF (96ORF78) that did not interact with DnaI was used as a negative control. Results shown are the means of quadruplicate data ± sd.

phages of *S. aureus* and identified a total of 31 distinct families of growth-inhibitory proteins. Several families contained highly related polypeptides from different phages (data not shown).

S. aureus DnaI is the cellular target of phage 77ORF104

The bacterial targets of phage ORF–induced growth inhibition were identified by affinity chromatography of *S. aureus* lysates and visualization of phage-associated proteins on polyacrylamide gels. A single polypeptide of 38 kDa was recovered specifically from an *S. aureus* lysate fractionated on an affinity column containing immobilized 77ORF104 (**Fig. 2a**). No polypeptide of this size was recovered from a glutathione *S*-transferase (GST) control column or from a 77ORF104 column without applied *S. aureus* extract. The identity of the 38 kDa polypeptide was revealed by trypsinolysis and mass spectrometry[25] to be the *S. aureus* homolog of *Bacillus subtilis* DnaI, a protein that is required for primosome assembly and which is essential during the initiation of DNA replication[26–28].

Validation of the interaction between DnaI and 77ORF104

In order to confirm that 77ORF104 associates specifically and directly with DnaI, we performed a series of cell-based and *in vitro* assays. First, the association between 77ORF104 and DnaI was confirmed in a yeast two-hybrid assay in which only coexpression of the two proteins allowed specific growth of *Saccharomyces cerevisiae* on selective medium (THAL–; **Fig. 2b**). This result was obtained whether 77ORF104 and DnaI were used as either bait or prey (data not shown).

Second, far-western analysis, in which a strong hybridization signal was detected between immobilized DnaI and ^{32}P-labeled 77ORF104 (**Fig. 2c**), confirmed the direct interaction of 77ORF104 with DnaI. Third, biomolecular interaction (BIAcore) analysis showed that the equilibrium dissociation constant (K_d) of the interaction between 77ORF104 and DnaI was approximately 51 nM (**Fig. 2d**). Last, a miniaturized solution-phase assay for 77ORF104-DnaI interaction based upon time-resolved fluorescence resonance energy transfer (TR-FRET) between europium cryptate and allophycocyanin[29] revealed an IC_{50} (concentration at which 50% of the signal is inhibited) of approximately 60 nM for untagged 77ORF104 (**Fig. 2e**), a result consistent with BIAcore data. This result provided the basis for a screen for small-molecule inhibitors of the interaction between 77ORF104 and DnaI (see below).

DnaI is an essential protein in *S. aureus*

To address the question of whether the protein targeted by 77ORF104 is dispensable, we assessed the essentiality of DnaI for the viability of

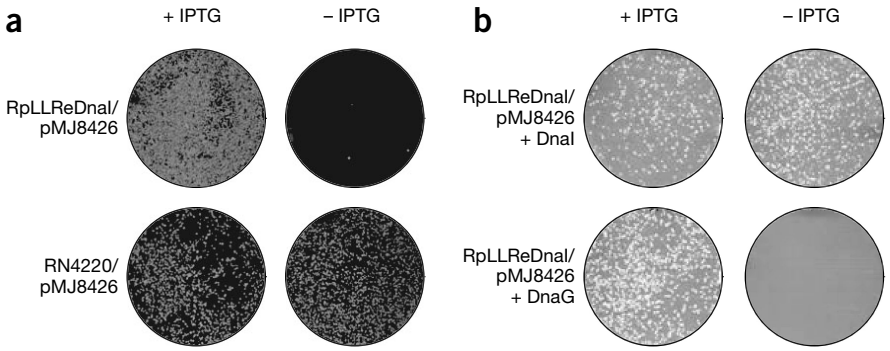


Figure 3 *dnaI* essentiality analysis. (**a**) RpLLReDnaI is a genetically modified *S. aureus* strain in which the expression of *dnaI* is under the control of the IPTG-inducible *spac* promoter. Plasmid pMJ8426, constitutively expressing LacI, was introduced into RpLLReDnaI and control strain RN4220. Transformants were plated on TSA containing 3 μg/ml tetracycline ± 0.3 mM IPTG. Strain RpLLReDnaI/pMJ8426 grows only in the presence of IPTG. (**b**) Transcomplementation. Strain RpLLReDnaI/pMJ8426 was transformed with a plasmid expressing either DnaI or DnaG of *S. aureus*. RpLLReDnaI/pMJ8426 grows in the absence of IPTG only when transcomplemented with a plasmid expressing DnaI.

S. aureus in laboratory culture conditions. Initial attempts to disrupt the *dnaI* gene in the *S. aureus* genome by an allele-replacement technique yielded no viable clones (data not shown), indicating that *dnaI* may be essential for bacterial growth. We confirmed the essentiality of *dnaI* by modifying *S. aureus* RN4220 using an inducible promoter replacement system[30], in which expression of chromosomal *dnaI* was driven by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *spac* promoter. Compared to the wild-type RN4220 strain, the growth of the modified strain RpLLReDnaI/pMJ8426 was strictly dependent on the presence of IPTG (**Fig. 3a**), suggesting that expression of *dnaI* is required for cell growth. To rule out possible polar effects within the *dnaI* operon, a transcomplementation experiment was conducted with DnaI expressed from a plasmid. Expression of a plasmid-borne copy of *dnaI* in the modified *S. aureus* strain RpLLReDnaI/pMJ8426 was able to compensate for IPTG-dependency (**Fig. 3b**). Taking all the results together, we conclude that *dnaI* is an essential gene of *S. aureus*.

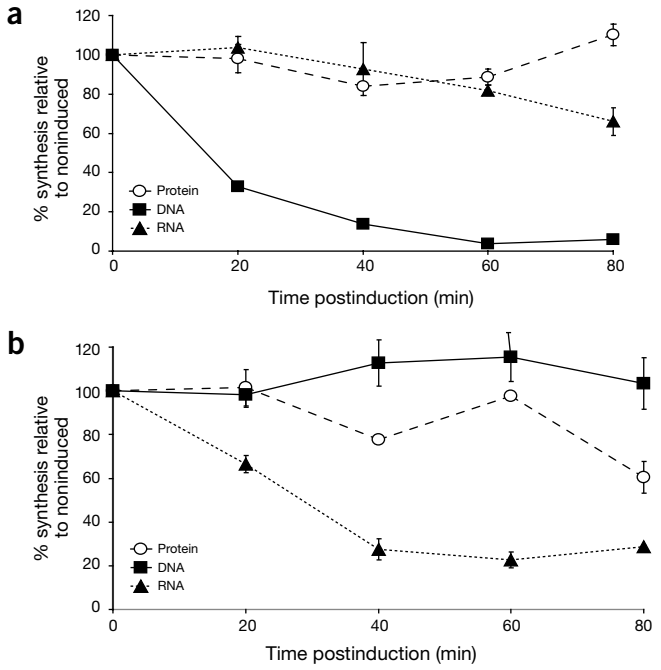


Figure 4 Effects of phage inhibitory ORF expression on metabolic pathways. (**a**) 77ORF104 inhibits DNA synthesis. (**b**) ORF67 inhibits RNA synthesis. Exponentially growing *S. aureus* RN4220 cells containing cloned phage ORFs under induced and uninduced conditions were pulse-labeled[39] at each time point with ^{3}H-thymidine (DNA), ^{3}H-uridine (RNA) or ^{35}S-methionine (protein) for 15 min. Each data point represents the average of two independent clones ± sd.

Expression of 77ORF104 inhibits DNA synthesis

Since DnaI is proposed to have a specific role in DNA replication, we investigated the selectivity of inhibition of DNA synthesis when 77ORF104 is expressed in *S. aureus*. Incorporation of ^{3}H-thymidine into DNA was markedly and selectively reduced in cells expressing 77ORF104 (**Fig. 4a**) but not in cells expressing a noninhibitory ORF (77ORF30; data not shown). No significant effect of 77ORF104 expression on RNA or protein synthesis was observed. In summary, our findings are consistent with the proposal that 77ORF104 mediates its growth-inhibitory effect in *S. aureus* through a direct and specific interaction with DnaI, the product of an essential gene. DnaI is thus a phage-validated target for drug discovery.

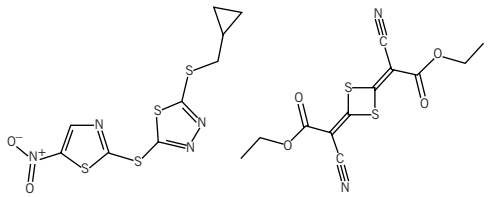
Compounds from screening have antimicrobial activity

The hypothesis that a small-molecule compound, selected to inhibit the interaction between a growth-inhibitory phage polypeptide and its cognate bacterial protein, could inhibit the growth of bacteria was thus tested as a prelude to drug discovery. We screened 125,000 small-molecule compounds from commercially available libraries in a fluorescence-based assay for inhibitors of the 77ORF104-DnaI interaction and identified 36 compounds with an IC_{50} of less than 10 μM for the interaction in a TR-FRET assay (**Fig. 2e**; controls shown). The ability of these compounds to inhibit bacterial growth, expressed as minimum inhibitory concentration (MIC), and their effect on DNA and RNA synthesis, were determined for *S. aureus*. Among the 36 compounds, 11 were found to have MIC ≤ 16 μg/ml. The characterization of two such compounds that were directly identified from the commercially available libraries is summarized in **Table 1**. Both compounds were found to inhibit DNA synthesis more than RNA synthesis in *S. aureus*. Furthermore, neither compound was significantly cytotoxic to human primary hepatocytes or to the cell lines Hep G2 and HeLa (data not shown).

Antimicrobial compounds act via DnaI

To investigate whether the antimicrobial activity of the compounds was due to direct action on their proposed molecular target, DnaI, we used the RpLLReDnaI/pMJ8426 strain that had been generated for the essentiality analysis of DnaI (see above). In this strain, expression of DnaI can be regulated through the use of varying concentrations of the inducer IPTG. The susceptibility of *S. aureus* strain

Table 1 Examples of two small molecules with antibacterial activity against *S. aureus*

	Compound 1	Compound 2
77ORF104-DnaI interaction		
IC_{50} (μM)[a]	1.5	4.8
MIC (μg/ml)[b]	0.125	16
DNA synthesis IC_{50} (μg/ml)	0.70	1.5
RNA synthesis IC_{50} (μg/ml)	1.5	14
MIC ratio (100/25 μM IPTG)[c]	4	8
Structure		

[a]IC_{50} values for the 77ORF104-DnaI interaction were determined in an *in vitro* assay based on TR-FRET between Eu^{3+} cryptate and allophycocyanin[29]. [b]MICs were determined using *S. aureus* strain RN4220 following NCCLS guidelines[38]. [c]MICs were determined using *S. aureus* strain RpLLReDnaI/pMJ8426 in TSB medium containing 3 μg/ml tetracycline and in the presence of 100 or 25 μM IPTG. For this strain, no MIC shift was observed for control antibiotics (norfloxacin, rifampicin, ampicillin, triclosan and gentamicin).

RpLLReDnaI/pMJ8426 to compounds active against DnaI will thus depend on the concentration of inducer. As predicted, a decrease in the concentration of IPTG from 100 to 25 μM resulted in a four- to eight-fold (75 to 87.5%) decrease in the observed MICs for the two example compounds (**Table 1**). In contrast, there was no observable difference in the MIC of known antibiotics (norfloxacin, rifampicin, ampicillin, triclosan and gentamicin) active against targets other than DnaI with *S. aureus* strain RpLLReDnaI/pMJ8426 under the same conditions (data not shown). A correlation between high and low inducer concentrations and the cellular DnaI level in strain RpLLReDnaI/pMJ8426 was observed on western blots of cell lysates with anti-DnaI antibodies (data not shown). Furthermore, no MIC shift or change in DnaI expression was observed for wild-type *S. aureus* strain RN4220/pMJ8426 under the same experimental conditions. Taken together with the results of macromolecular synthesis inhibition, these observations suggest that the two example compounds inhibit bacterial growth via DnaI and thus via DNA replication.

DISCUSSION

Using the bacteriophage genomics approach described here, we found that four proteins of the *S. aureus* DNA replication machinery were targeted by a total of seven unrelated phage polypeptides (**Table 2**). Concordantly, these seven phage polypeptides selectively inhibited ^{3}H-thymidine incorporation in *S. aureus* (**Fig. 4a** and data not shown). Our finding that two proteins of the DNA replication complex, DnaI and DnaN, are each targeted by multiple unrelated phage polypeptides (**Table 2**), as was seen for the replicative helicase DnaB in *E. coli*[12,13], suggests that these essential proteins are readily accessible to inhibition in *trans* by large molecules such as phage polypeptides. Importantly, our study further demonstrates that the small-molecule compounds identified through high-throughput screening for inhibitors of the ORF-target interaction possess antimicrobial activity via their intended target. Despite many potential target proteins in the DNA replication pathway, only topoisomerases are targeted by currently available antibiotics[31]. The phage genomics approach described here allows the prioritization of additional DNA replication proteins for drug discovery.

Table 2 *S. aureus* DNA replication proteins identified by antimicrobial phage ORFs

Representative of inhibitory ORF family[a]	ORF size (aa)	Bacterial target identified	Function of target	Essentiality of target[b]
77ORF104	52	DnaI	Helicase loader	Essential
ORF016	297			
ORF025	58	DnaN	DNA Pol III	Essential
ORF168	74		β subunit	
ORF240	58			
ORF078	71	DnaG	DNA Primase	Essential
ORF140	101	PT-R14	Involved in DNA replication	Not determined

[a]Amino acid sequences of phage ORFs are unrelated between families. Except for ORF240 and ORF140, ORFs are derived from different *S. aureus* phages. All ORFs specifically inhibited DNA synthesis in *S. aureus*. [b]Determined by allele replacement/disruption and IPTG-inducible gene regulation (data not shown).

The phage genomics platform described here also facilitated the identification of proteins in other metabolic pathways within *S. aureus*, including transcription. As an example, inhibitory ORF67 from a phage isolated in-house selectively inhibited RNA synthesis (**Fig. 4b**); its cognate cellular target is a component of the transcriptional machinery and is essential for bacterial viability (data not shown).

In summary, we present a systematic and high-throughput phage genomics approach to identify phage ORFs with antimicrobial activity and their cognate bacterial target proteins for subsequent drug discovery. These targets are evolutionarily validated to be susceptible to inactivation by antimicrobial phage ORFs. Our approach allows us to identify and prioritize bacterial proteins such as DnaI that, as far as we know, are not currently pursued by the industry as targets for antibiotic development. Our platform also provides a ready-to-use screening assay based on inhibition of protein-protein interactions between a phage inhibitory ORF and its cognate bacterial target. Small-molecule inhibitors that were identified through such a screen with DnaI displayed antimicrobial activity via this intended target. For some of our systems, deletion analysis allowed the identification of the minimal interacting domain between the phage ORF and its cognate bacterial target (data not shown). These domains may represent the sites of interaction of the phage ORFs and may therefore be used to augment the specificity and selectivity of the screen for small-molecule inhibitors in drug discovery.

We are expanding this phage genomics platform to other bacterial pathogens, including *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*, and this should allow the development of a wide variety of small-molecule inhibitors for the control of current and future microbial pathogens.

METHODS

Propagation of *S. aureus* bacteriophages and genome sequencing. *S. aureus* propagating strains and bacteriophages were obtained from several sources including the National Collection of Type Cultures, England; Laboratory Center for Disease Control, Canada; American Type Culture Collection; and H.-W. Ackermann of the Centre de Référence pour Virus Bactériens, Canada. Isolation and propagation of phages and preparation of phage genomic DNA followed published protocols[32,33]. Phage genomic DNA was randomly cleaved by sonication and the ends were repaired with T4 DNA polymerase and Klenow fragment and cloned into the *Hin*cII site of plasmid pKS (Stratagene). The inserts were sequenced using BigDye primer or BigDye terminator cycle sequencing reactions (ABI Prism). Sequence contigs were assembled using

Sequencher 3.1 (GeneCodes) or PhredPhrap/Consed 12.0 (CodonCode Corporation) software. Each genome was sequenced at least once in each direction. The assembled contigs had at least threefold coverage obtained from independent clones.

Genomics and functional genomics analysis of phage ORFs in *S. aureus*. Bacteriophage ORFs were identified as follows: beginning at the first nucleotide, the phage genome sequence is scanned for a start codon. When one is identified, the number of in-frame codons is counted until a termination codon is reached. A minimum threshold of 33 codons defines this bounded sequence as an ORF. This procedure is repeated, starting at the next nucleotide following the last stop codon, until the end of the phage sequence is reached. The scan is performed in an identical manner on all three reading frames of both DNA strands of the phage sequence, in order to identify all the putative ORFs. Putative genes are then identified based on the presence of a Shine-Dalgarno sequence within the 15 nucleotides upstream of the start codon. Putative genes were predicted based on the presence of a Shine-Dalgarno sequence within 15 bp upstream of the start codon. This software will be made available upon request. The *E. coli-S. aureus* shuttle vector pTOO21 (ref. 20), containing the arsenite-inducible *ars* promoter and the *arsR* gene, was modified with an optimal Shine-Dalgarno sequence (AGGAGG) followed by a multiple cloning site (MCS). DNA encoding individual phage ORFs was amplified by PCR from phage genomic DNA and cloned into the MCS of the modified vector. Recombinant plasmids were introduced into *S. aureus* RN4220 by electroporation[34] and clones were selected on tryptic soy agar plates containing 30 μg/ml kanamycin (TSA/Kan). Phage ORFs that inhibited the growth of *S. aureus* were identified in a dot screen on TSA/Kan ± 5 μM $NaAsO_2$. Inhibitory ORFs were further characterized in growth kinetics assays as described in the legend to **Figure 1c** using tryptic soy broth containing 30 μg/ml kanamycin (TSB/Kan) ± 5 μM $NaAsO_2$.

Identification of bacterial targets of phage-encoded growth inhibitory ORFs. Phage-encoded inhibitory polypeptides were expressed as GST or His_6 fusions and purified from *E. coli*. Recombinant proteins were covalently coupled to affigel resin (BioRad) at protein/resin concentrations ranging from 0 to 5 mg/ml using HNG buffer (20 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 10% (vol/vol) glycerol). Unreacted sites were blocked with 10 mM ethanolamine, pH 7.5 for 30 min at 4 °C. Lysates were prepared from exponential-phase *S. aureus* cells (3 g) resuspended in 20 ml of HNG buffer (1 mM each of dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF)), and protease inhibitor cocktail (Roche Diagnostics), 20 μg/ml each of RNase A and DNase I, and lysed either with 1,250 Units of lysostaphin (Sigma) followed by sonication, or mechanically with a Bead-Beater (Biospec Products) according to the manufacturer's instructions. The lysed cell suspension was made up to 500 mM NaCl, 1 mM EDTA and 1% (vol/vol) Triton X-100 and was mixed for 30 min at 4 °C. Cell debris was removed by ultracentrifugation for 3 h at 93,000*g* and the supernatant was dialyzed overnight at 4 °C against affinity chromatography (AC) buffer (20 mM Hepes-KOH, pH 7.5, 10% (vol/vol) glycerol, 1 mM DTT, 1 mM EDTA) containing 100 mM NaCl and 1 mM PMSF and stored in aliquots at –80 °C. Affinity chromatography was performed by mixing 40 μl of resin, crosslinked to either phage polypeptide or GST as a control, with 400 μl of *S. aureus* lysate for 30 min at 4 °C. The resin was washed extensively with AC buffer containing increasing concentrations (0.1–1 M) of NaCl. Bound proteins were eluted with 1% (wt/vol) SDS, resolved by SDS-PAGE and stained with silver nitrate. Specific bands were excised and proteins were digested with trypsin and subjected to matrix-assisted laser desorption ionization/mass spectrometry in conjunction with post-source decay and collision-induced decay for determination of protein identity[25].

Confirmation of phage polypeptide/bacterial protein interaction. Yeast two-hybrid analysis was performed using the Matchmaker Two-Hybrid System 3 (CLONTECH Laboratories) following the manufacturer's protocol. SV40 large T-antigen and Lamin C were used as controls for autoreactivity. Far-western analysis was performed as described[35]. Briefly, 77ORF104 fused with a heart muscle kinase site[36] was radiolabeled and used as a probe to detect DnaI immobilized on a nitrocellulose membrane. BIAcore analysis was performed on a BIAcore 2000 instrument using an anti-GST sensor chip (BIAcore) with approximately 20 resonance units (RU) of GST-tagged 77ORF104 captured as ligand. A control surface without captured ligand was used for reference subtraction. For kinetic analysis, DnaI protein at different concentrations was injected in duplicate over the GST-77ORF104 surface (3 min association, 15 min dissociation). The data were analyzed using numerical integration[37]. For the TR-FRET assay, proteins and fluor-conjugated antibodies were diluted in TR-FRET buffer (20 mM Hepes, pH 8.0, 400 mM KF, 1 mM EDTA, 0.1% (wt/vol) bovine serum albumin, 0.01% (vol/vol) Tween 20). Final protein and antibody concentrations for the assay were as follows: His-tagged DnaI and GST-tagged 77ORF104, 10 nM; anti-His-europium cryptate (CIS-US), 2 nM; anti-GST-allophycocyanin (Prozyme), 33 nM in a final assay volume of 30 μL.

Gene essentiality analysis of *DnaI*. The gene essentiality analysis of *dnaI* done following the procedure described by Jana *et al*[30]. Specifically, a 0.88 kb fragment upstream of *dnaI* and a 0.91 kb *dnaI* fragment starting from its ribosome binding site were amplified by PCR from *S. aureus* RN4220 genomic DNA and sequentially cloned and inserted into the MCS of pLL2443 vector. The resultant plasmid, which contains the two PCR fragments flanking the Cat-Tl $ter_{(5)}$-p*Spac* cassette was used to transform *S. aureus* strain RN4220 to allow homologous recombination. Resolution of the integrant resulted in the genetically modified strain RpLLReDnaI in which chromosomal *dnaI* is under the control of the *spac* promoter. This strain was validated by PCR, sequencing and Southern blot analysis. To regulate DnaI expression in RpLLReDnaI, plasmid pMJ8426, which constitutively expresses the *E. coli lacI* gene, was introduced into RpLLReDnaI. The essentiality of DnaI was then evaluated by comparing overnight bacterial growth of RpLLReDnaI/pMJ8426 on TSA plates containing 3 μg/ml tetracycline ± 0.3 mM IPTG. Wild-type RN4220 was used as a control.

To rule out the possibility that polar (neighboring gene) effects were responsible for the essential phenotype of RpLLReDnaI/pMJ8426 in the absence of IPTG, a transcomplementation experiment was conducted. Strain RpLLReDnaI/pMJ8426 was transformed with a plasmid expressing either DnaI or DnaG of *S. aureus*. Overnight bacterial growth on TSA plates containing 3 μg/ml tetracycline and 30 μg/ml kanamycin was then compared with growth in the presence of 0.3 mM IPTG.

Screening for inhibitors of phage polypeptide–bacterial protein interaction. Small-molecule compounds were screened at 20 μM in 10 mM HEPES pH 8.0, 0.005% Tween 20, 50 mM NaCl, 40 μg/mL bovine gamma globulin, 2% dimethyl sulfoxide in a fluorescence polarization assay which used Oregon Green 488 (λEX, 496 nm; λEM, 524 nm; Molecular Probes)-labeled 77ORF104 protein (8 nM, final concentration) as tracer and DnaI protein (300 nM, final concentration) as binder. Proteins were incubated with compounds for 2 h at ambient temperature (22 to 25 °C) and fluorescence polarization was determined in 384-well Proxiplates (Packard) using an Ultra fluorescence plate reader (Tecan).

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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