Revealing bacterial targets of growth inhibitors encoded by bacteriophage T7

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Today's arsenal of antibiotics is ineffective against some emerging strains of antibiotic-resistant pathogens. Novel inhibitors of bacterial growth therefore need to be found. The target of such bacterial-growth inhibitors must be identified, and one way to achieve this is by locating mutations that suppress their inhibitory effect. Here, we identified five growth inhibitors encoded by T7 bacteriophage. High-throughput sequencing of genomic DNA of resistant bacterial mutants evolving against three of these inhibitors revealed unique mutations in three specific genes. We found that a nonessential host gene, *ppiB*, is required for growth inhibition by one bacteriophage inhibitor and another nonessential gene, *pcnB*, is required for growth inhibition by a different inhibitor. Notably, we found a previously unidentified growth inhibitor, gene product (Gp) 0.6, that interacts with the essential cytoskeleton protein MreB and inhibits its function. We further identified mutations in two distinct regions in the *mreB* gene that overcome this inhibition. Bacterial two-hybrid assay and accumulation of Gp0.6 only in MreB-expressing bacteria confirmed interaction of MreB and Gp0.6. Expression of Gp0.6 resulted in lemon-shaped bacteria followed by cell lysis, as previously reported for MreB inhibitors. The described approach may be extended for the identification of new growth inhibitors and their targets across bacterial species and in higher organisms.

target identification | host takeover | bacterial cytoskeleton | bacteriophage biology | high-throughput DNA sequencing

Bacteria have evolved to overcome a wide range of antibiotics; in some bacteria, the resistance mechanisms against most conventional antibiotics have been identified (1, 2). This increasing threat is spurring the identification of novel antimicrobials against novel molecular targets in the pathogens (e.g., refs. 3–6). There are currently only a few host molecules targeted by antibiotics. These targets (and examples of the antibiotics against them) are host RNA polymerase (rifampicin), topoisomerase (quinolones), cell wall (penicillin), membranes (polymyxin), ribosome (tetracyclines, aminoglycosides, macrolids), and synthesis of nucleic-acid precursors (sulfonamides, trimethoprim). Increasing the arsenal of bacterial targets and antimicrobial drugs against them is valuable, and novel strategies to increase this repertoire are therefore of great importance.

One strategy for the identification of novel antibacterial targets is to determine how bacteriophages shut down their host's biosynthetic pathways and enslave its machinery during infection. Phages have coevolved with bacteria for over 3 billion years and have thus developed molecules to specifically and optimally inhibit or divert key metabolic functions. Examples of bacterial targets inhibited by phage-derived products include the δ subunit of the DNA polymerase III clamp loader, inhibited by gene product (Gp) 8 of the coliphage N4 (7); the *Staphylococcus aureus* putative helicase loader, DnaI, inhibited by ORF104 of bacteriophage 77 (5); a key enzyme of folate metabolism, FolD, inhibited by Gp55.1 of the coliphage T4 (8); and the essential cell-division protein, filamenting temperature-sensitive mutant Z (FtsZ), inhibited by Gp0.4 of the coliphage T7 (9). These examples suggest that there are other phage products that may inhibit other bacterial targets.

A model for the systematic study of host–virus interactions and for elucidating phage antibacterial strategies is provided by bacteriophage T7 and its host, *Escherichia coli*. The laboratory strain *E. coli* K-12 shares many essential genes with pathogenic species, such as *E. coli* O157:H7 and O104:H4, and therefore, growth inhibitors against it should prove effective against these pathogens as well. *E. coli* has been studied extensively, and the putative functions or tentative physiological roles of over half of its 4,453 genes have been identified. T7 is a virulent phage that upon infection of its host, *E. coli*, produces over 100 progeny phage per host in less than 25 min. It is an obligatory lytic phage, and therefore, its successful growth cycle always results in lysis of the host. Despite extensive knowledge of the T7 phage, the mechanism by which it manipulates host functions remains obscure. Specific functions have been attributed to over half of the 56 T7 Gps (9–12); all of the phage structural Gps are well characterized, as are those Gps that take part in phage DNA replication. However, most of the remaining Gps that take over the host machinery have not yet been characterized, and the host proteins with which they interact have not been identified. We hypothesized that some of these Gps would inhibit *E. coli* growth by targeting specific essential proteins.

Here we propose an approach to searching for antibacterial targets using whole-genome DNA sequencing. The basic underlying principle is that many resistance mutations against growth inhibitors arise in target genes. Therefore, by expressing

Significance

Antibiotic resistance of pathogens is a growing threat to human health, requiring immediate action. Identifying new gene products of bacterial viruses and their bacterial targets may provide potent tools for fighting antibiotic-resistant strains. We show that a significant number of phage proteins are inhibitory to their bacterial host. DNA sequencing was used to map the targets of these proteins. One particular target was a key cytoskeleton protein whose function is impaired following the phage protein's expression, resulting in bacterial death. Strikingly, in over 70 y of extensive research into the tested bacteriophage, this inhibition had never been characterized. We believe that the presented approach may be broadened to identify novel, clinically relevant bacteriophage growth inhibitors and to characterize their targets.

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a growth inhibitor and identifying resistance mutations using whole-genome sequencing, one may be able to identify its target. High-throughput sequencing has been recently used to identify genetic interactions (e.g., refs. 13–15) but not host–virus interactions. Advances in DNA sequencing technology, as well as its accessibility and affordability, are enabling its application to the identification of bacterial targets at high throughput and minimal cost. We used this approach to search for bacterial targets of T7 bacteriophage proteins. We cloned most of the uncharacterized genes of T7 bacteriophage and tested their inhibition of bacterial growth. We then isolated mutants that are resistant to these growth inhibitors and identified the arising mutations by high-throughput sequencing. The mutations arose multiple times, in unique genes for each growth inhibitor, indicating a unique mechanism for overcoming the inhibition. Of particular interest was inhibition of the essential cytoskeleton protein, MreB, which was further validated by genetic and biochemical methods.

Results

High-Throughput Sequencing Identifies a Characterized Inhibitor–Target Interaction. We hypothesized that expression of growth inhibitors in *E. coli* would result in resistance mutations in the target genes and that these mutations could be identified using high-throughput DNA sequencing of the genomes of the selected resistant mutants (Fig. 1). To test the feasibility of this approach, we expressed the growth inhibitor Gp0.4 of T7 bacteriophage, which inhibits the division protein FtsZ (9). Twelve mutants resistant to this growth inhibitor were isolated, and their genome was deep-sequenced. We expected to identify a specific mutation that renders FtsZ refractory to Gp0.4 inhibition and hence enables survival of the bacteria encoding this mutation, as we previously reported (9). Indeed, following analysis of the sequencing results (described in the next section), we identified a unique mutation encoded only by resistant mutants expressing Gp0.4. Other genes were also mutated in these resistant mutants, but none were unique, as they were also mutated in mutants resistant to other growth inhibitors. The specific mutation was an insertion mutation of 6 nt in *ftsZ*, shown to confer resistance to Gp0.4 expression (9). The frequency of this mutation was 61.54%, suggesting it appears in the majority of the 12 resistant mutants (Table 1). As expected, this mutation in *ftsZ* was only observed in mutants resistant to the Gp0.4 growth inhibitor, and not in other resistant mutants. This finding validated the feasibility of the approach for specifically identifying novel targets of phage-derived growth inhibitors.

High-Throughput Sequencing Reveals Targets of Growth Inhibitors. To search for targets of novel growth inhibitors, we cloned 14 uncharacterized genes from T7 phage on a plasmid: genes 0.5, 0.6, 1.1, 1.4, 1.5, 1.6, 1.8, 2.8, 3.8, 4.1, 4.2, 4.3, 4.7, and 5.3. These genes were cloned downstream of an L-arabinose–inducible promoter, plated in the presence of D-glucose (transcription repressor) or L-arabinose (transcription activator), and bacterial viability was monitored in both cases. All bacteria encoding these 14 genes grew well on plates supplemented with 0.2% (wt/vol) D-glucose. However, among these 14 genes, five were inhibitory to the host upon L-arabinose induction—genes 0.6, 1.6, 3.8, 4.3, and 5.3—whereas the other nine were not inhibitory (Fig. 2). To identify the Gps targeted by these five growth inhibitors, we expressed them in *E. coli* and isolated 15–83 independent resistant mutants from each of the five transformants encoding the growth inhibitors (Table S1). In all cases, resistant mutants emerged at a frequency of $\sim 2 \times 10^{-7}$. To discriminate between genomic mutations and mutations occurring in the growth inhibitor itself, we excluded mutants in which the plasmid lost its inhibitory effect due to disruptive mutations in the growth inhibitor. To this end, we extracted plasmids from those colonies that have become resistant to the phage inhibitors, retransformed them into another strain, and checked their inhibition. We found that all of the resistant mutants formed in response to expression of Gp1.6 or 5.3 evolved from mutations in the plasmid (Table S1), and these mutants were eliminated from further studies. Genomic DNA was extracted from mutants resistant to genes 0.4 (positive control), 0.6, 3.8, and 4.3, whose plasmids retained growth inhibition, and sequenced using an IlluminaHiSEq. 2500. The sequencing results were analyzed by applying several filters. Mutations occurring in less than 5% of the reads were discarded. Mutations passing this filter were further processed only if more than 90% of the mutations in a single gene were uniquely mapped to a specific group of resistant mutants (Table 1). As described above, this analysis identified FtsZ as the top target of Gp0.4, as expected. It also identified the accumulation of missense mutations in *mreB*, encoding the cytoskeleton protein MreB, in mutants resistant to Gp0.6. Moreover, it determined that disruptive mutations in genes *ppiB* and *pcnB* accumulate in mutants resistant to Gp 3.8 and 4.3, respectively (Table 1). Note that the above filters yielded a unique mutated gene for every group of resistant mutants, and each was found to confer resistance, as described in the following sections.

Nonessential Targets PpiB and PcnB Validated as Contributing to Inhibitor Functionality. Both *ppiB* and *pcnB* encode nonessential proteins, and therefore, resistance could be validated using *E. coli* lacking these genes. We transformed the plasmids encoding genes

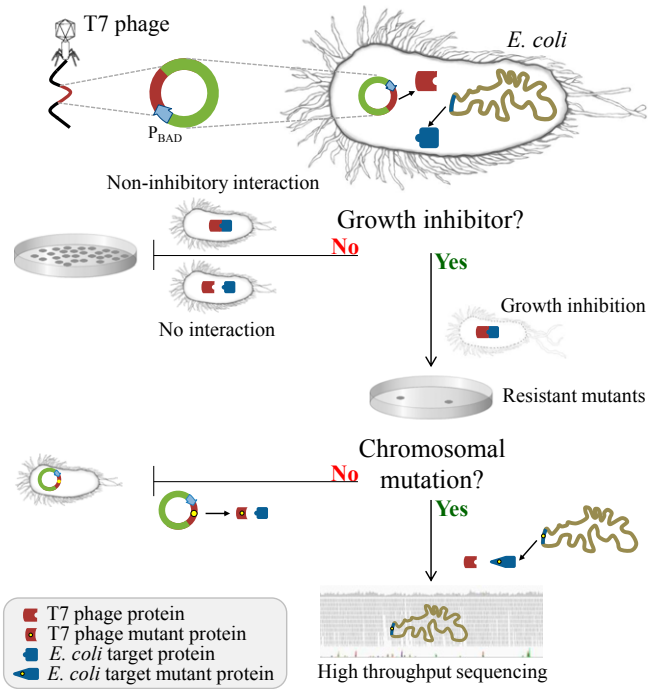

Fig. 1. Schematic representation of the approach used to identify novel targets of bacteriophage growth inhibitors. *E. coli* bacteria are transformed with plasmids cloned with genes derived from T7 bacteriophage downstream of an inducible promoter (P_{BAD}). Expression of noninhibitory genes results in viable bacteria, and these genes are excluded from further analysis. Expression of inhibitory genes results in resistant mutants. These resistant mutants are isolated and then tested for plasmid growth inhibition. Plasmids are extracted from these clones, and their growth inhibition is validated by retransformation into *E. coli* bacteria, as described in *SI Materials and Methods*. Mutants whose plasmids have lost growth inhibition are excluded from further analysis. Mutants whose plasmids are inhibitory are suspected of having mutations in the genome that confer resistance. Genomes of these mutants are extracted, sequenced, and analyzed.

Table 1. Resistant mutants revealed by high-throughput sequencing

Mutated host gene	Mutation type	Mutation[a]	Expressed toxic gene			
			Gp0.4	Gp0.6	Gp3.8	Gp4.3
ftsZ	Insertion	105351	61.54[b]	<5	<5	<5
ftsZ	Mismatch	105363	7.32	<5	<5	<5
ftsZ	Mismatch	106107	5.80	<5	<5	<5
mreB	Mismatch	3398235	<5	12.00	<5	<5
mreB	Mismatch	3398242	<5	8.22	<5	<5
mreB	Mismatch	3398244	<5	5.48	<5	<5
mreB	Mismatch	3398246	<5	9.46	<5	<5
mreB	Mismatch	3398247	<5	5.41	<5	<5
mreB	Mismatch	3398635	<5	19.05	<5	<5
mreB	Mismatch	3398649	<5	20.73	<5	<5
mreB	Mismatch	3398649	<5	9.76	<5	<5
mreB	Mismatch	3398649	<5	7.32	<5	<5
ppiB	Deletion	553223	<5	<5	12.12	<5
ppiB	Deletion	553226	<5	<5	8.91	<5
ppiB	Deletion	553302	<5	<5	13.92	<5
ppiB	Deletion	553342	<5	<5	7.25	<5
ppiB	Deletion	553377	<5	<5	6.41	<5
ppiB	Deletion	553378	<5	<5	9.88	<5
ppiB	Mismatch	553427	<5	<5	7.69	<5
ppiB	Deletion	553452	<5	<5	6.67	<5
ppiB	Insertion	553461	<5	<5	6.33	<5
pcnB	Deletion	158087	<5	<5	<5	7.69
pcnB	Mismatch	158090	<5	<5	<5	8.16
pcnB	Mismatch	158091	<5	<5	<5	9.28
pcnB	Insertion	158432	<5	<5	<5	7.62
pcnB	Mismatch	158504	<5	<5	<5	11.76
pcnB	Mismatch	158631	<5	<5	<5	5.61
pcnB	Mismatch	158645	<5	<5	<5	9.00
pcnB	Mismatch	158769	<5	<5	<5	7.23
pcnB	Mismatch	158888	<5	<5	<5	5.15

[a]Numbering refers to *E. coli* strain K-12, accession no. NC_000913.
[b]Percent mutations.

3.8 and 4.3 into strains deleted in *ppiB* and *pcnB*, respectively. These transformants were then plated on medium having 0.2% D-glucose or 0.2% (wt/vol) L-arabinose to control expression of the growth inhibitor. Indeed, as expected, lack of PpiB conferred resistance to Gp3.8 expression (Fig. 3*A*), and lack of PcnB conferred resistance to Gp4.3 expression (Fig. 3*B*). A positive control, demonstrating that both Gp3.8 and Gp4.3 inhibit growth in these settings, was carried out in an isogenic strain, whose growth was inhibited in the presence of L-arabinose, as expected. These results indicated that Gp3.8 and Gp4.3, respectively, require the *ppiB* and *pcnB* genes or their products for growth inhibition. One possibility for the growth inhibition is that Gp3.8, a putative homing endonuclease (10), or Gp4.3 inhibits growth by cleaving unique restriction sites in the *ppiB* or *pcnB* genes, respectively. In this scenario, the growth inhibitors cleave the chromosome at these genes, but deletion of these genes eliminates the restriction sites from the chromosome, thus rescuing the bacteria. If this is the case, then complementing the deletion mutants with plasmids encoding *ppiB* and *pcnB* genes should not restore growth inhibition by Gp3.8 and Gp4.3. To test this, we transformed *E. coli* mutants lacking *ppiB* with plasmids expressing PpiB and Gp3.8 or expressing PpiB and a control plasmid. Similarly, we transformed *E. coli* mutants lacking *pcnB* with plasmids expressing PcnB and Gp4.3 or expressing PcnB and a control plasmid. We then monitored growth in the presence of transcription repressor (D-glucose) or inducer (L-arabinose) of the growth inhibitors. Notably, at this stage, we did not add antibiotics to which the *ppiB* or *pcnB* encoding plasmids confer resistance, and therefore, plasmid cleavage and consequent loss should not result in bacterial death. We observed that plasmids encoding the *ppiB* and *pcnB* genes did restore growth inhibition in the respective deletion mutants (Fig. 3 *C* and *D*). The control strains encoding only the complementing genes did not inhibit growth, indicating that toxicity is not due to their expression but rather due to the growth inhibitors. This result shows that growth inhibition in the presence of the respective growth inhibitors results from the interactions with the PpiB and PcnB proteins and rules out the possibility that growth inhibition is caused by DNA cleavage of unique restriction sites located in the genes encoding them. Taken together, these results validated our approach and expanded its original scope to the identification of nonessential genes that mediate growth inhibition of some growth inhibitors. A putative mechanism by which these nonessential genes mediate the growth inhibition is proposed in *Discussion*.

MreB Validated as the Target of Phage Inhibitor Gp0.6. The third identified putative target was *mreB*. Because MreB is essential to the host (16), validation of the interactions could not be carried out by its deletion. We first validated the high-throughput sequencing results by Sanger sequencing of the 10 independent resistant clones. This procedure identified 10 independent mutations in the *mreB* gene (Table S2). These mutations were in agreement with the deep-sequencing analysis. Notably, the mutations were clustered

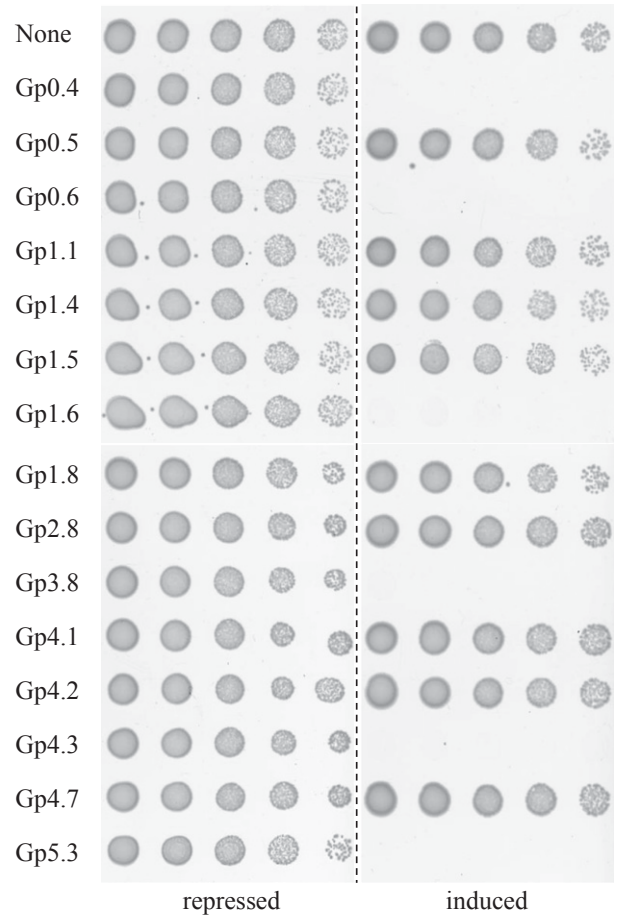

Fig. 2. Identification of T7 bacteriophage Gps inhibiting bacterial growth. *E. coli* NEB5α bacteria transformed with a plasmid encoding the indicated Gp were serially diluted in 10-fold increments. These dilutions (highest dilution on the *Right*) were then inoculated on LB agar supplemented with 0.2% D-glucose (repressed) or 0.2% L-arabinose (induced). Images of LB agar plates represent one out of two experiments showing similar results.

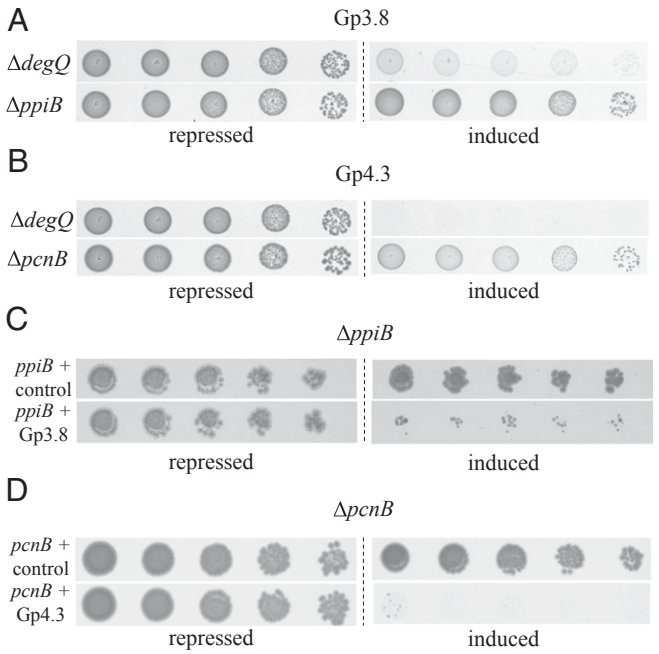

Fig. 3. Requirement of *ppiB* and *pcnB* for inhibition by Gp3.8 and Gp4.3, respectively. *E. coli* bacteria lacking the indicated gene and transformed with a plasmid encoding either Gp3.8 (*A*) or Gp4.3 (*B*) were serially diluted in 10-fold increments. These dilutions (highest dilution on the *Right*) were then inoculated on LB agar supplemented with 0.2% D-glucose (repressed) or 0.2% L-arabinose (induced). Deletion mutants of *ppiB* (*C*) or *pcnB* (*D*) were transformed with plasmids encoding the indicated genes. These transformants were serially diluted in 10-fold increments, and dilutions (highest dilution on the *Right*) were then inoculated on LB agar supplemented with 0.2% D-glucose (repressed) or 0.2% L-arabinose (induced). Images of LB agar plates represent one out of two experiments showing similar results.

into two main segments, 154–159 and 288–292, indicating that the possible binding pocket(s) of Gp0.6 to MreB is near the residues encoded by these mutations. These results confirmed that resistance mutations to Gp0.6 expression accumulate in *mreB*.

To validate experimentally that the mutations in *mreB* alone confer resistance to Gp0.6 expression, we constructed a strain carrying a mutation conferring resistance to Gp0.6 expression, MreB^{E288G}. The mutation was generated by isolation of the Gp0.6-resistant mutant, followed by transduction of the mutated *mreB* gene into fresh *E. coli* culture (see *SI Materials and Methods* for details). Colonies transduced with this mutation or control transductants were tested for resistance to Gp0.6 by transforming them with the inducible plasmid encoding Gp0.6 and plating on Luria–Bertani (LB) medium supplemented with L-arabinose. Three clones carrying the *mreB* mutation were refractory to Gp0.6, whereas three clones lacking the mutation remained sensitive to Gp0.6 (Fig. 4*A*). These results confirmed that the mutation E288G in *mreB* is sufficient to render the cell resistant to Gp0.6 growth inhibition.

Gp0.6 and MreB Interact in the Bacterial Cell. To genetically test whether Gp0.6 and MreB interact, we used the bacterial two-hybrid system, a bacterial version of the yeast two-hybrid assay (17). This system enables the identification of protein–protein interactions in vivo. *mreB* was fused, in frame, to one domain, T18, of the cAMP cyclase gene. Gene 0.6 was fused, in frame, to another domain, T25, of the cAMP cyclase gene. Under these conditions, the cAMP cyclase enzyme is activated only if the two domains are brought into close proximity by the interactions of the two tested proteins. Its activity results in LacZ induction, which is easily detected as blue-colored colonies on LB plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). As a positive control, we used the two domains, each fused to the leucine zipper domain (ZIP). As a negative control, we used two proteins that do not interact—MreB fused to the T18 domain and ZIP fused to the T25 domain. *E. coli* cotransformed with the indicated plasmids were plated on LB plates supplemented with the appropriate antibiotics and X-gal. Cotransformation of plasmids encoding MreB fused to T18 and Gp0.6 fused to T25 resulted in LacZ activity similar to the positive control (Fig. 4*B*). As expected, the negative control yielded no LacZ activity. These results indicated that MreB and Gp0.6 interact in vivo.

To further validate these interactions, we attempted to carry out a pull-down assay. Crude extracts were prepared from bacteria cotransformed with the Gp0.6-encoding plasmid along with plasmids encoding either MreB or a control protein, CheZ. Notably, Gp0.6 was detected by Western blot only in bacteria

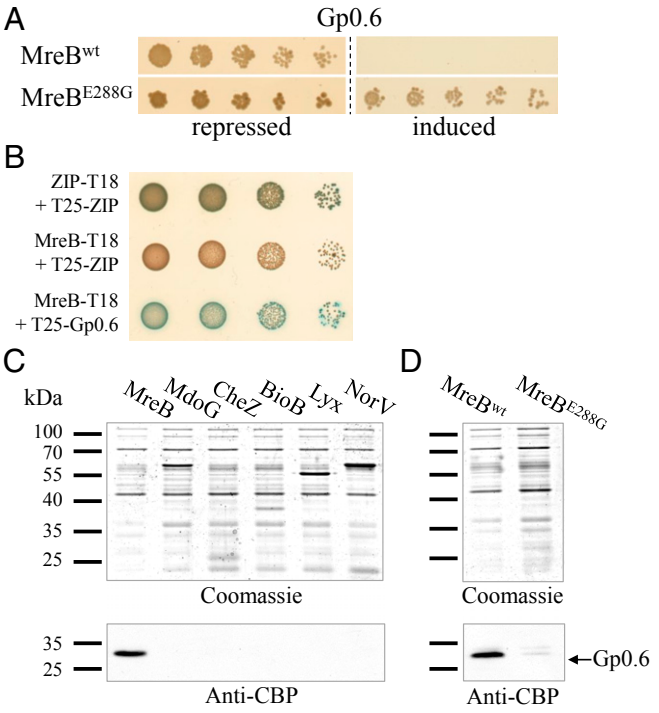

Fig. 4. Validation of Gp0.6 interaction with MreB. (*A*) *E. coli* bacteria encoding either wild-type MreB (MreB^{wt}) or MreB^{E288G} transformed with a plasmid encoding Gp0.6 were serially diluted in 10-fold increments. These dilutions (highest dilution on the *Right*) were then inoculated on LB agar supplemented with 0.2% D-glucose (repressed) or 0.2% L-arabinose (induced). (*B*) *E. coli* bacteria resistant to Gp0.6 inhibition (encoding MreB^{E288G}) were cotransformed with the indicated plasmids and serially diluted in 10-fold increments. These dilutions (highest dilution on the *Right*) were then inoculated on LB agar supplemented with X-gal and incubated for 24 h. (*C*) *E. coli* bacteria resistant to Gp0.6 inhibition (encoding MreB^{E288G}) harboring a plasmid encoding a calmodulin-binding peptide (CBP)-tagged Gp0.6 were cotransformed with plasmids encoding the indicated his$_6$-tagged proteins. Protein expression was induced by addition of 0.2% L-arabinose and 1 mM IPTG. Whole-cell lysates were then prepared, and a sample was electrophoresed on a 12% (wt/vol) polyacrylamide gel. Coomassie staining to validate equal loading of the purified proteins (*Top*) and Western blot using anti-CBP to detect Gp0.6 (*Bottom*) were carried out. (*D*) *E. coli* bacteria resistant to Gp0.6 inhibition (encoding MreB^{E288G}) or encoding the MreB^{wt} were transformed with a plasmid encoding CBP-tagged Gp0.6. Gp0.6 expression was induced by addition of 0.2% L-arabinose. Coomassie staining to validate equal loading (*Top*) and Western blot using anti-CBP to detect Gp0.6 (*Bottom*) were carried out. Gel and plate images represent one out of at least two experiments showing similar results.

expressing the MreB but not CheZ (Fig. 4*C*). This surprising result prevented a controlled pull-down assay as the level of Gp0.6 was significantly different between the samples. However, it showed that the increase in the steady-state level of Gp0.6 was specific to MreB expression. To strengthen this finding, we showed that Gp0.6 did not accumulate in *E. coli* cotransformed with plasmids encoding four other randomly chosen proteins (Fig. 4*C*). Furthermore, the steady-state level of Gp0.6 was detectible only in *E. coli* encoding the $mreB^{wt}$ gene in its chromosome and not in an isogenic strain encoding a Gp0.6-resistant $mreB^{E288G}$ allele (Fig. 4*D*). Thus, the specific accumulation of Gp0.6 only in strains encoding a Gp0.6-sensitive MreB protein further confirmed the specific MreB's interaction with Gp0.6.

MreB Inhibition by Gp0.6 Changes Bacterial Morphology. Inhibition of MreB is morphologically manifested as "lemon-shaped" bacteria (18). We therefore expected that if the binding of Gp0.6 to MreB was inhibitory, this morphology would be seen. We also expected that bacteria refractory to Gp0.6 growth inhibition would show normal morphology following Gp0.6 expression. Indeed, light microscopy showed a typical lemon shape for *E. coli* bacteria harboring the plasmid encoding Gp0.6 after 2 h of 0.2% L-arabinose induction (Fig. 5*A*). In contrast, the morphology of *E. coli* encoding a resistant *mreB* mutant was not affected by Gp0.6 expression, as expected. Moreover, as reported for MreB inhibition (18, 19), many cells expressing the wild-type MreB underwent lysis during that time (Fig. 5*B*). Altogether, these results indicated that Gp0.6 inhibits MreB function in vivo.

Discussion

We demonstrated that high-throughput DNA sequencing of resistant bacterial mutants can identify the targets of uncharacterized growth inhibitors or reveal proteins that mediate their inhibition. Unique mutations corresponding to single genes were found in mutants resistant to each of the four tested growth inhibitors—one that had been previously reported and three unreported phage-derived growth inhibitors.

Deletion of the nonessential gene *ppiB* eliminated Gp3.8 growth inhibition. PpiB is a cytoplasmic peptidyl-prolyl cis-trans isomerase involved in protein folding (20). We speculate that this protein is involved in folding of the Gp3.8 growth inhibitor, and thus in its absence, Gp3.8 is not folded into its active state, resulting in a noninhibitory protein.

Another nonessential gene, *pcnB*, eliminated the inhibitory effect of Gp4.3. PcnB has been shown to maintain high copy numbers of plasmids, and therefore, lack of this protein probably results in decreased copy number of the plasmid encoding the growth inhibitor. This is likely the reason for the reduced growth inhibition by Gp4.3 (21). Indeed, disruption of *pcnB* reduced growth inhibition also by Gp3.8 (Fig. S1), suggesting that Gp3.8 also requires high plasmid copy number to exert its inhibition. Nevertheless, in the initial high-throughput sequencing analysis, we did not find *pcnB* disrupted in response to the expression of Gp3.8. A possible explanation for this result is that alternative mutations in *ppiB*, which overcome growth inhibition by Gp3.8, are more frequent than *pcnB* mutations. Growth inhibition by Gp0.4, 0.6, 1.6, and 5.3 was not significantly reduced in the absence of *pcnB* (Fig. S1) probably because they inhibit growth even at medium and low doses, and therefore, reduced plasmid copy number does not alleviate their inhibition.

An essential Gp that may serve as a novel target for antibiotics is MreB, which was inhibited by Gp0.6. We showed that at least 11 different mutations can render MreB resistant to Gp0.6 inhibition. The fact that we obtained only one mutation twice whereas the other mutations were represented once suggests that there are other mutations that alleviate MreB inhibition by Gp0.6. The obtained mutations were clustered in two regions, suggesting that Gp0.6 binds to MreB in the pockets formed by these encoded regions. We further demonstrated that Gp0.6 binds to the wild-type MreB and that expression of Gp0.6 in *E. coli* results in a phenotype that is characteristic of MreB inhibition. As an inhibitor, Gp0.6 can be used to study bacterial cytoskeleton arrangement, and it can also potentially serve as a new tool in the fight against antibiotic-resistant bacteria. From a therapeutic viewpoint, MreB is a possible target for antibiotics, as it is an essential bacterial protein that is conserved across most rod-shaped bacteria and absent in eukaryotes (16, 22). Indeed, some chemical compounds known to depolymerize MreB filaments have been suggested as antibacterial agents (e.g., refs. 23–25). Further studies on Gp0.6 to facilitate its use as an antimicrobial compound should determine the minimum effective peptide length for inhibition, its MreB-inhibition capability across pathogenic bacterial species, its stability inside and outside mammalian tissues, and its penetration efficiency into the bacteria.

What does the phage gain from inhibiting MreB by Gp0.6? Under standard laboratory conditions (LB medium, 37 °C, and aerated culture), we could not detect any significant advantage for T7 phages that encode Gp0.6 versus those that lack it. Nevertheless, under other as-yet unidentified conditions, this gene's inhibition might be useful for phage growth. It is intriguing that the newly identified inhibitor of MreB, Gp0.6, is encoded near Gp0.4 (203 bp away), a recently identified inhibitor of FtsZ (9). MreB and FtsZ are key cytoskeletal proteins in *E. coli*. They are responsible for proper chromosome segregation and movement, as well as for cell-wall integrity during elongation and division (22, 26–29). Interestingly, two proteins of *Bacillus* Φ29 phage interact with FtsZ and MreB, but in contrast to the T7 proteins, they do not inhibit them but rather exploit them for phage replication (30). The fact that FtsZ has been shown to confer a competitive advantage to the phage merely via inhibition of FtsZ suggests that this is also the case for MreB. We speculate that inhibiting MreB, a protein that is important for cell-wall stability and maintenance (22, 26, 27), results in

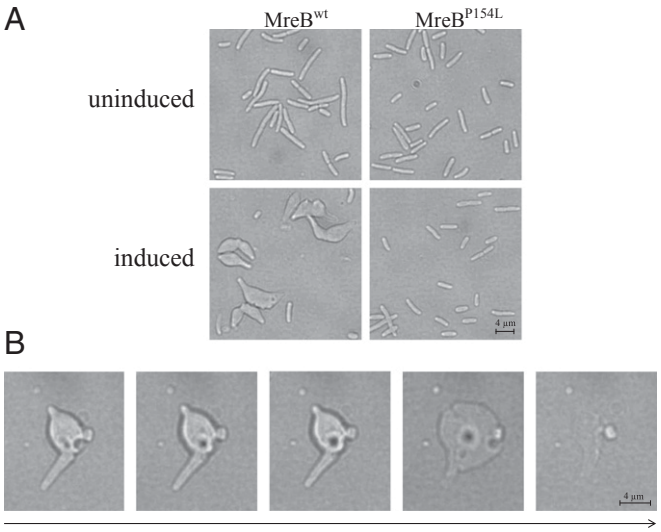

Fig. 5. Morphology of *E. coli* expressing Gp0.6. (*A*) *E. coli* encoding the wild-type MreB protein ($MreB^{wt}$) or the Gp0.6-resistant protein $MreB^{P154L}$ harboring the pBAD33-Gp0.6 plasmid were induced with 0.2% L-arabinose (induced) to drive expression from the plasmid promoter or left uninduced (uninduced), as described in *SI Materials and Methods*. Images were taken after 2 h of growth. (Scale bar, 4 μm.) Images represent one out of 10 experiments showing similar results. (*B*) A single $MreB^{wt}$ bacterium expressing Gp0.6 for ~2 h is shown in images taken at five 1-s intervals. Arrow indicates order of events. This series of images represents one out of dozens observed.

loosening of the cell wall, which in turn allows smoother release of new virions. This speculation is supported by our observation that bacterial lysis is induced by MreB inhibition by Gp0.6, but direct evidence is required to establish this. Also worth noting is the fact that an *E. coli* growth inhibitor, YeeV, manifests inhibition of both FtsZ and MreB in the same polypeptide (18). This single-polypeptide structure, which simultaneously inhibits both MreB and FtsZ, suggests that Gp0.4 and Gp0.6 perform similar functions and are thus encoded from a single operon in the bacteriophage genome.

Taken together, the results presented in this article show that using the described approach, it is possible to search for novel bacterial growth inhibitors and their targets. The finding that a significant proportion (five out of 14) of the uncharacterized phage products inhibited growth of their host highlights this approach's potential for finding more such products in other bacterium–phage systems. The robustness of the approach is emphasized by the fact that it revealed an inhibitor that had remained unknown during almost 70 y of extensive research into the T7 phage. In addition to targeted essential genes, the approach also identified nonessential genes that mediate the growth inhibition.

Despite the robustness of the approach, it has some limitations. If the mutation rate in the chromosome is significantly less than that of the growth inhibitor encoded on the plasmid, it becomes difficult to obtain chromosomal resistance mutants that maintain plasmid growth inhibition. This situation could occur in cases where multiple mutations are required to overcome growth inhibition—for example, if the target is not a single gene, but rather a complex, membrane, cell wall, DNA, or RNA. Depending on the frequency of the mutation in the genome compared with that on the plasmid, it may occasionally be possible to select resistant mutants in such cases as well, after thorough screening. Alternatively, two different plasmids encoding the same growth inhibitor can be cotransformed into the bacteria, thus reducing the probability of growth inhibitor loss that would have to occur simultaneously on both plasmids. Another downside of our approach is that the optimal therapeutic agents are those to which resistance forms at low frequency—that is, those that are best masked from this approach. Nevertheless, despite this issue, we believe that identifying several such antibacterial substances and combining them in a mixture should overcome the formation of simultaneous resistance against all of them. Moreover, the main advantage of this approach is that it can recognize targets of uncharacterized antibacterial substances and may elucidate novel pathways and mechanisms operating in the cell, as demonstrated in this study. These results, in turn, may pave the way for the structural design of more potent inhibitors of those targets, leading to effective antibacterial substances.

Materials and Methods

The reagents, bacterial strains, phages, plasmids, and oligonucleotides used in this study are listed in *SI Materials and Methods* and Table S3. Plasmid construction, growth inhibition assays, other genetic and biochemical assays, as well as the isolation of resistant mutants, analysis of the high-throughput data, and microscopy are all described in *SI Materials and Methods*.

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