

An array of target-specific screening strains for antibacterial discovery

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As the global threat of drug- and antibiotic-resistant bacteria continues to rise, new strategies are required to advance the drug discovery process. This work describes the construction of an array of *Escherichia coli* strains for use in whole-cell screens to identify new antimicrobial compounds. We used the recombination systems from bacteriophages λ and P1 to engineer each strain in the array for low-level expression of a single, essential gene product, thus making each strain hypersusceptible to specific inhibitors of that gene target. Screening of nine strains from the array in parallel against a large chemical library permitted identification of new inhibitors of bacterial growth. As an example of the target specificity of the approach, compounds identified in the whole-cell screen for MurA inhibitors were also found to block the biochemical function of the target when tested *in vitro*.

The development of a new antibiotic is ultimately a futile endeavor¹. Because bacteria are formidably adaptive organisms, heritable resistance will always evolve with time and selection pressure from antibiotic exposure². Ironically, the use of an antibiotic leads to its obsolescence, as the rate of resistance development is proportional to the frequency of its use¹. Every antibiotic placed into clinical use has engendered resistance, irrespective of its chemical class or molecular target³. Increased antibiotic treatment in animal husbandry and fish farming has only accelerated the selection process⁴. An evolved resistance phenotype can spread both clonally, because of improved fitness of the resistant strain, and across species, as bacteria readily transmit genetic material that encodes resistance determinants⁵. As current antibiotic therapy grows increasingly ineffectual, it is critically important to isolate structurally novel antibiotics that forestall the emergence of acquired resistance. However, except for linezolid (an oxazolidinone launched last year that has already encountered clinical resistance⁶), no new chemical class of antibiotic has been introduced into clinical practice for over 30 years. Why has the pharmaceutical industry failed to produce novel antibiotics⁷?

All clinically useful antibiotics, including linezolid, were identified originally as agents that possessed antibacterial activity. Such broad cell-based screening does not identify the biochemical target of a lead compound, hindering efforts to optimize the compound on the basis of structure–activity relationships. To overcome this obstacle, the pharmaceutical industry changed its antimicrobial discovery approach in the early 1980s from screening for antibacterial activity to inhibiting specific biochemical targets. Although this strategy has produced many potent enzyme inhibitors, it has yet to yield a clinically useful antibiotic⁸. In fact, the search for a target-specific inhibitor is a profound challenge. Because slight structural changes are likely to decrease the exquisite potency of the inhibitor, there is little room to maneuver when trying to endow the molecule with other desirable properties, such as permeability, broad-spectrum

antibacterial activity, oral bioavailability, and metabolic stability. Unable to make any progress in the development process, investigators abandon the inhibitor (and often the target), along with the chance of producing a new drug. Cycling through this discovery process one target at a time is inefficient, especially in view of the multitude of valid essential targets for antibiotic development^{9,10}.

We report here a different approach to discovering lead compounds that inhibit specific essential targets, have sufficient pharmaceutical potential, and, most importantly, have antibacterial activity. In an industrial environment in which chemistry and development resources are limited, a key aim of any antibacterial drug discovery program is to identify such a compound quickly. Merging the ideas of target-based discovery with the techniques of current bacterial genetics allows a directed, yet inexpensive and technically simple approach to whole-cell screening.

We engineered an array of bacterial strains to be more sensitive for specific enzyme inhibitors than for antibacterial activity alone. We achieved this by first cloning the essential gene for a specific target under control of a highly regulated promoter on a plasmid. This plasmid then served as the complementing copy for the essential gene, which was removed from the genome by recombination using linear DNA transformation. What remained was a bacterial cell in which the intracellular level of one essential target protein could be adjusted with a specific inducer. After modulating target concentration in the bacterium, the strain was screened against a chemical library using growth inhibition as an end point.

Using this strategy, it is possible to find leads that not only are active against whole cells but also are specific for a molecular target. Assaying a large number of modified bacterial strains against the same collection of compounds permits the compilation of a two-dimensional database in which one can query the activity of a specific compound for all strains screened. Thus one can identify compounds with good inhibitory activity against more than one

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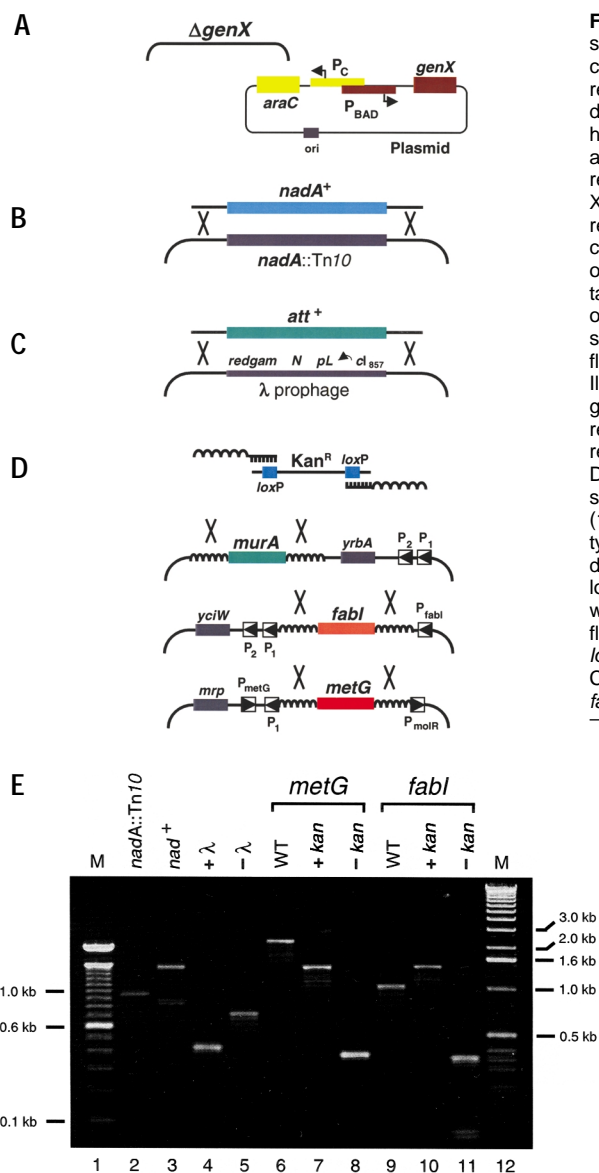


Figure 1. Construction of *E. coli* strains for parallel screening. (A) Plasmid-based strategy for regulated expression of a target gene that has been deleted from the chromosome (bent lines). P_C , P_{BAD} , promoters of the *araC* and *araBAD* gene clusters, respectively; *genX*, any bacterial gene X. The plasmid origin of replication (*ori*) is denoted by the small shaded box and represents any plasmid origin (from low copy to high copy number). (B) Recombination using linear, double-stranded DNA molecules and λ -*redgam*. In this example, the gene for nicotinamide adenine dinucleotide A replaces the mutant version on the chromosome. Blunt-ended lines, linear DNA; large X, λ -*red*-mediated crossover between regions of homologous DNA. (C) Linear DNA recombination to replace the defective λ prophage. *att*⁺ refers to a fragment of *E. coli* chromosomal DNA containing the bacteriophage λ attachment site, *attB*. (D) Deletion of target genes on the chromosome using the *lox2*-*kan* cassette. Colored boxes, target genes (*murA*, *fabI*, and *metG*); shaded boxes, neighboring genes that encode open reading frames; curvy lines, homologous DNA shared by the linear DNA substrate and the bacterial chromosome. P_1 and P_2 designate promoter elements flanking the target genes; specific promoters for each target are identified by name. Illustrations are not to scale. (E) Agarose gel showing PCR products generated from genomic DNA templates of various *E. coli* strains constructed with the bacteriophage recombination systems. Lanes 1 and 12: marker DNA (0.1 kb and 1 kb ladders, respectively). Lanes 2 and 3: removal of transposon Tn10 from chromosome of DY329; a multiplex PCR using primers within *tetA* and flanking *nadA* shows that the smaller internal band (0.95 kb) is lost when the wild-type *nadA* allele is restored (1.45 kb band, lane 3). The faint 0.85 kb band (lane 3) is a nonspecific PCR product typical of multiplex PCR. Lanes 4 and 5: removal of the λ prophage; a multiplex PCR done with primers flanking and within the prophage shows the smaller internal band is lost after the wild-type *attB* allele is restored. For the *metG* deletion, PCR was done with an internal primer (directed against the *kan* coding sequence) and two primers flanking *metG*. Lanes 6–8: PCR products representing *metG*, replacement by *lox2*-*kan* cassette, and the PCR product after removal of the kanamycin marker by P1 Cre, respectively. Lanes 9–11: corresponding PCR products with primers flanking the *fabI* coding sequence. Experimental details available upon request.

bacteriophage P1 for removal of selectable markers to minimize misregulation and polar effects (Fig. 1D). Cre recombinase binds to specific sequences adjacent to *lox* sites and removes the intervening DNA (ref. 13).

Initially we used these phage recombination systems to construct reference strains for parallel screening. We first converted a defective λ -prophage strain inducible for *redgam* (DY329) to *nad*⁺ by transformation with appropriately designed linear DNAs generated through PCR (Fig. 1B). PCR products were synthesized with primers that amplified the *nadA* region from a wild-type strain. Selection of *nad*⁺ prototrophs resulted in loss of the proximal Tn10 transposon and its associated resistance marker, *tetA*. Replica plating and PCR confirmed loss of tetracycline resistance (Fig. 1E, lanes 2 and 3). From this strain, the chromosomal copy of the arabinose regulon (including *araB*, *araA*, and *araD*, which encode the arabinose-metabolizing enzymes) was deleted from its normal location at 1.5 min using a similar strategy (unpublished data). Deletion of the *ara* regulon is required for uncomplicated regulation by arabinose when the P_{BAD} promoter is fused to target genes on a plasmid¹⁴. In addition, *pcnB*, a host gene that controls plasmid copy number of ColE1 origins¹⁵, was deleted using similar methods. Thus, we constructed two *ara*⁻ *E. coli* strains (*pcnB*⁺ and *pcnB*⁻) to serve as: (i) parent strains for further genetic manipulations and (ii) reference strains for screening.

Table 1. Gene targets, their protein products, and functions in *E. coli* for regulated expression and parallel screening

Gene	Product	Function
<i>dnaB</i>	Helicase	DNA replication
<i>fabI</i>	Enoyl-ACP reductase	Fatty acid biosynthesis
<i>folA</i>	Dihydrofolate reductase	Intermediary metabolism
<i>gyrB</i>	DNA gyrase, subunit B	DNA replication
<i>metG</i>	Methionyl-tRNA synthetase	Translation
<i>murA</i>	UDP-acetyl-D-glucosamine enolpyruvyl transferase	Cell wall biosynthesis
<i>pyrH</i>	UMP kinase	Intermediary metabolism
<i>tufA(B)</i>	Elongation factor Tu	Translation
<i>ycdO</i>	41 kDa protein	Unknown

essential target, so-called multimodal inhibitors. Such compounds would have lower frequencies of acquired resistance^{2,11}. Finally, it is feasible to consider other desirable pharmaceutical properties of the leads (metabolic stability, toxicity, oral bioavailability, bactericidal mode of action, slow or fast killing) to determine development priorities. In short, this strategy allows the assessment of which lead compounds have the best properties to determine which antibacterial targets are worthy of more intensive biochemical analysis.

Results

Construction of screening strains. To achieve regulated expression of essential target genes in *E. coli*, we deleted the chromosomal copy of the gene and expressed the complementary copy from the arabinose regulon on a plasmid (Fig. 1A). As described later, we constructed chromosomal deletions and allelic exchanges using the generalized recombination system from bacteriophage λ (*redgam*). When λ -*redgam* functions are expressed, linear DNA molecules introduced into *E. coli* are protected from degradation and serve as substrates for homologous recombination¹². Following allelic exchange, we used the Cre/*lox* site-specific recombination system of

Table 2. Susceptibility of engineered strains to known inhibitors

		Engineered strains								
Inhibitors	Reference strain	↓ <i>murA</i>		↓ <i>fabI</i>		↓ <i>tufA</i>		↓ <i>metG</i>		[Ara]
		Low	High	Low	High	Low	High	Low	High	
Ampicillin	2.0	2.0	2.0	ND	ND	1.0	1.0	4.0	4.0	
Bacitracin	8.0	8.0	8.0	ND	ND	ND	ND	ND	ND	
Fosfomycin	0.25	0.03	>8.0	0.125	0.125	ND	ND	0.5	0.5	
Fusidic acid	1,000	ND	ND	ND	ND	500	1000	ND	ND	
Kanamycin	1.6	ND	ND	0.8	1.6	4.0	4.0	4.0	4.0	
Kirromycin	128	ND	ND	ND	ND	8	64	ND	ND	
Norfloracin	0.05	0.05	0.05	0.05	0.05	ND	ND	0.05	0.05	
Rifampicin	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	
Triclosan	0.1	ND	ND	0.05	0.4	ND	ND	ND	ND	

Minimum inhibitory concentration ($\mu\text{g/ml}$) of known agents for each strain tested in microtiter plates in LB broth at 37°C. Reference strain values are independent of arabinose concentration. For the $\downarrow tufA$ strain, both the *tufA* and *tufB* alleles were deleted from the chromosome with the complementing plasmid containing *tufA* only. The low- and high-arabinose concentrations (shown as "low" and "high" in the table) used for each engineered strain are as follows: 1.6 μM and 13 mM for the $\downarrow murA$ strain (seed culture at 1.3 mM), 65 μM and 13 mM for the $\downarrow fabI$ strain (seed culture at 65 μM), 520 μM and 13 mM for the $\downarrow tufA$ strain (seed cultures at 520 μM), and 1.3 mM and 13 mM for the $\downarrow metG$ strain (seed cultures at 130 μM). ND, not determined.

To construct target-specific screening strains, we amplified a kanamycin-resistance gene cassette flanked by *loxP* sites^{16,17} by PCR using primers with long tails (~50 base pairs). The tails share homology with regions immediately upstream and downstream of an essential target gene (Fig. 1D). Direct transformation of the PCR product into cells expressing *redgam* followed by kanamycin selection results in replacement of the target gene with the *lox²-kan* cassette. When the target is essential for bacterial growth, 100% of the transformants show the appropriate deletion only if the plasmid copy of the gene is induced (data not shown). PCR confirmed the location of the allele exchange on the chromosome (Fig. 1E, lanes 7 and 10).

Using the site-specific recombinase from bacteriophage P1, we removed the *kan* cassette from deletion strains. We transiently expressed the P1 Cre protein (cloned into a low-copy-number, temperature-sensitive plasmid) in cells, and subsequently eliminated the plasmid by incubation at 37°C. The result was excision of the antibiotic-resistance marker, leaving a single *lox* site in its place¹³. Replica plating and PCR confirmed loss of kanamycin resistance (Fig. 1E, lanes 8 and 11).

In the last step before screening, we removed the temperature-sensitive λ prophage from each strain. λ^- recombinants were selected at 42°C following transformation of a 4 kilobase PCR fragment generated from an *att⁺* strain (Fig. 1C). PCR (Fig. 1E, lanes 4 and 5) and phage plating (data not shown) confirmed removal of the λ_{857} prophage. A partial list of target-specific strains engineered by this methodology is shown in Table 1.

Characterization of hypersusceptible strains. Reducing expression of an essential gene should affect a cell's growth characteristics and render it hypersusceptible to specific inhibitors. As a first test of this hypothesis, we analyzed the growth rate of the engineered strains in microtiter plates. A matrix of conditions was developed to monitor the effects of arabinose concentration, inoculum size, and inhibitor concentration. Results in Figure 2 show that both $\downarrow metG$ and $\downarrow fabI$ strains were similarly affected by lowering arabinose concentrations. (A downward arrow (\downarrow) directly preceding a gene name indicates a bacterial strain in which expression of that gene is under inducible control.) Growth defects appeared as a delayed escape from lag phase followed by exponential growth comparable to the reference strain. In fact, doubling times at low and high inducer concentrations were nearly identical (2.5–3.0 h). At 65 μM arabinose, exponential growth of the $\downarrow fabI$ strain began only after an 8 h delay.

In contrast, the $\downarrow metG$ strain showed a similar delay, but at 420 μM arabinose. The conditions necessary to demonstrate significant growth defects were unique for each strain and required experimental determination (Fig. 2).

At the lowest inducer concentrations, growth rate drops off significantly for both the $\downarrow metG$ and $\downarrow fabI$ strains. Doubling time for the $\downarrow metG$ strain increased from 3 h (at 420 μM arabinose) to 5 h at 70 μM arabinose. A nearly identical increase in doubling time was seen for the $\downarrow fabI$ strain (3 h at 65 μM , 5 h at 6.5 μM).

For the $\downarrow murA$ strain, reducing arabinose did not lengthen the period before exponential growth until the lowest concentrations of inducer were tested. There was, however, a slight reduction in growth rate at 1.3 mM arabinose (3.7 h compared with ~3 h for

the reference strain). Lag phase was extended only after a tenfold reduction in inducer concentration. Lowering arabinose concentration to 13 μM affected both lag and exponential growth (as seen for $\downarrow fabI$ and $\downarrow metG$). When cultures were prepared under optimal conditions, none of the engineered strains were able to grow in the absence of inducer (Fig. 2).

From these data, we chose growth conditions for screening. We tested each target-specific strain for hypersusceptibility to known antibacterial agents by monitoring changes in the minimum inhibitory concentration (MIC; Table 2). *murA* is the target of the cell wall biosynthesis inhibitor, fosfomycin¹⁸. Arabinose-dependent downregulation of *murA* shifts the MIC for fosfomycin at least eightfold to 0.03 $\mu\text{g/ml}$ (as compared with the reference strain). High expression of MurA results in a >32-fold increase in the fosfomycin MIC (Table 2). Other antibiotics (for instance, norfloracin and trimethoprim) do not exhibit such a shift. Likewise, the $\downarrow fabI$

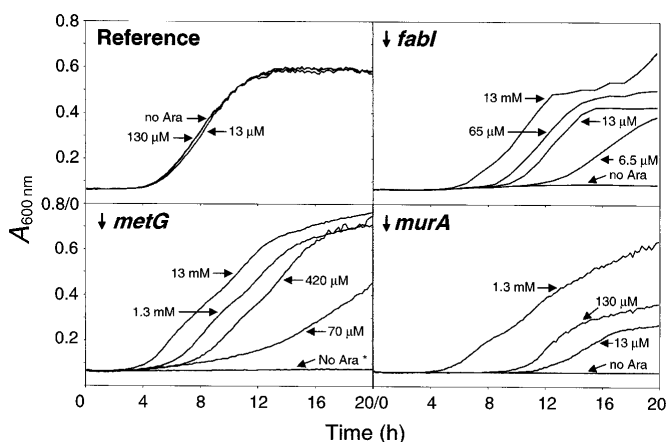


Figure 2. Kinetic growth analysis of engineered strains. Graphs plot $A_{600\text{ nm}}$ versus time for cultures in LB broth at 37°C (100 μl microtiter wells). Strain designations shown in each panel represent the name of the target gene engineered for regulated expression. Arabinose concentrations are indicated next to each curve. Seed cultures were grown with arabinose at the following concentrations: no arabinose for the reference strain, 0.13 mM for the $\downarrow fabI$ strain, 1.3 mM for the $\downarrow metG$ strain (* denotes seed culture grown in 0.13 mM arabinose), and 13 μM for the $\downarrow murA$ strain (in a *pcnB⁻* host). Refer to Experimental Protocol for details.

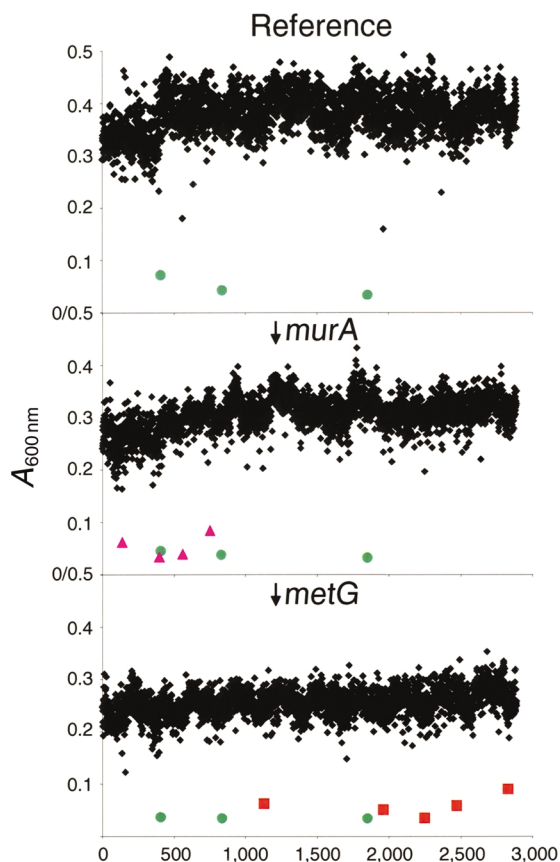


Figure 3. Cell-based parallel screening of engineered *E. coli* strains. Graphs plot $A_{600\text{nm}}$ versus compound number for cultures in LB broth at 37°C (100 μl microtiter wells). Reference strain was screened in the absence of inducer, while the $\downarrow\text{murA}$ and $\downarrow\text{metG}$ strains were screened under low-arabinose conditions. Hits common to all three strains are indicated by green circles (●). Hits specific for the $\downarrow\text{murA}$ and $\downarrow\text{metG}$ strains are indicated by pink triangles (▲) and orange squares (■), respectively.

and $\downarrow\text{tufA}$ strains demonstrate a MIC shift only with their cognate inhibitors: triclosan¹⁹ and kirromycin²⁰, respectively. For the $\downarrow\text{metG}$ strain, no shift in MIC was detected upon testing the strain against commercially available inhibitors of bacterial growth (Table 2).

Parallel high-throughput screening (HTS). We screened the reference strains against a compound library in 96-well microtiter plates using 100 μl Luria–Bertani (LB) broth containing 1% dimethyl sulfoxide at 37°C. Plates were read by absorbance at 600 nm after overnight incubation. Hits scored positive if the $A_{600\text{nm}}$ value was <0.1, and no visible growth could be detected in a well. Figure 3 shows the results from screening a reference strain against a minor fraction of compounds from our library. Three compounds from this set were able to prevent the growth of the *E. coli* reference strain (green circles). Upon screening the same set of compounds against the $\downarrow\text{murA}$ and $\downarrow\text{metG}$ strains, we identified the same three compounds (Fig. 3). In addition, four hits were found that were specific for the $\downarrow\text{murA}$ strain (pink triangles). Most striking is the identification of five new hits specific only to the $\downarrow\text{metG}$ strain (orange squares). These compounds were unable to prevent growth of either the reference strain or the $\downarrow\text{murA}$ strain.

Hits from the primary screens were retested for antibacterial activity using the engineered strains grown under different concentrations of inducer. If a hit is target-specific, upregulation of the target (by increasing inducer concentration) should render the bacteria less sen-

Table 3. HTS confirmation and target specificity

Compound	MIC				IC ₅₀
	Low [Ara]		High [Ara]		MurA Enzyme
	($\mu\text{g/ml}$)	(μM)	($\mu\text{g/ml}$)	(μM)	
1	0.6	(1.9)	9.8	(30)	2.0
2	3.4	(7.5)	27	(60)	1.4
3	5.2	(15)	>35	(>100)	6.2
4	6.2	(15)	>42	(>100)	2.9
5	2.8	(7.5)	11	(30)	2.4
Fosfomycin	0.08	(0.5)	>8	(>50)	0.2
Norfloxacin	0.05	(0.16)	0.10	(0.32)	ND

The first column lists a subset of active compounds from HTS of the $\downarrow\text{murA}$ strain. MIC values (in $\mu\text{g/ml}$) are shown at low (1.6 μM) and high (13 mM) concentrations of inducer (arabinose). Values in parentheses are micromolar MICs. Seed cultures of the $\downarrow\text{murA}$ strain were grown at 0.13 mM. The fourth column shows the concentration of compound required to inhibit the *in vitro* activity of the MurA enzyme by 50% (IC₅₀). ND, not determined.

sitive to the compound. Data from such a secondary screen appear in Table 3. We determined the absolute MICs for five compounds identified as hits in HTS of the $\downarrow\text{murA}$ strain. When the screening strain was grown at high inducer concentrations, the MICs for the compounds increased 16-fold for compound 1, 8-fold for compound 2, and 6-fold for both compound 3 and compound 4. Only a marginal shift in MIC is recorded for compound 5. For comparison, fosfomycin shows an increase of 100-fold under these same conditions. No change in the MIC for norfloxacin was recorded. When tested in an *in vitro* assay, these five compounds also inhibited the enzymatic activity of MurA (Table 3). IC₅₀ values for the compounds ranged from 1.4 to 6.2 μM . The IC₅₀ of fosfomycin is 0.2 μM (Table 3).

Discussion

We describe here the development and application of a screening strategy for antimicrobial drug discovery. This approach combines the attributes of more traditional discovery approaches^{21–23} into a simple, efficient, and high-throughput methodology that incorporates target specificity and antimicrobial activity into a primary screen. The cornerstone of this approach is the ability to generate an array of bacterial strains engineered for low-level expression of a particular essential gene. Each strain is then screened against a chemical library, using growth inhibition as an end point.

Strain construction was facilitated by the use of the recombination systems of bacteriophages λ and P1. The λ -*redgam* system is highly efficient, and allows the use of linear DNA molecules as recombination substrates^{24,25}. This avoids cumbersome genetic manipulations, such as repetitive plasmid cloning steps and the use of counterselectable markers to resolve recombination intermediates. In fact, recombination mediated by λ -*redgam* was highly reliable, often yielding 100% recombination efficiencies (data not shown). The portability of the *redgam* prophage was highly advantageous. It allowed the use of plasmid-based systems for controlled expression of target genes, providing flexibility with regard to incompatibility groups and selectable markers.

Removal of the prophage from the chromosome of each engineered strain prevented λ functions from interfering with screening (Fig. 1D). During HTS at 37°C, expression of λ -*kil* and other potentially toxic λ products should be repressed by the λ repressor, *cI*. Temperature variations can, however, occur in incubators harboring large numbers of multiwell plates. A 2°C increase in temperature (to 39°C) is known to induce the *cI*₈₅₇ temperature-sensitive allele²⁶. Killing as a result of prophage expression and the potential of false positives during screening necessitated removal of the prophage before HTS.



Expression of essential target genes was controlled by the well-characterized arabinose regulon²⁷. We chose episomal expression of target genes as a general strategy (Fig. 1A). Fusion of P_{BAD} to coding sequences on the chromosome was avoided because the majority of bacterial genes are found in operons. Such a strategy might subject flanking genes to arabinose control and misregulation, calling into question the specificity of any active compounds identified during HTS.

Plasmid-based expression has its advantages. Using plasmids not only precludes interference with expression of neighboring genes, but also provides an additional level of expression control—either by using plasmids with different copy numbers or by using mutants in nonessential host genes that alter plasmid copy number. In fact, the *pcnB* host was required to render \downarrow *murA* hypersusceptible for HTS (Figs 2, 3; Table 2). One drawback of using episomally expressed genes may be sensitivity to inhibitors of plasmid replication. Such inhibitors would show up as false positives during HTS, but would be easily sorted out by secondary testing under high-expression conditions (Table 3). In addition, inhibitors of plasmid replication would consistently score positive during the screening of many hypersusceptible strains, and would be easily identified by a routine analysis of the array HTS database.

As an additional precaution against polar effects and misregulation, we removed the antibiotic-resistant cassette used for allelic exchange from the chromosome. Presence of the *kan* cassette had the potential to alter the expression of neighboring genes and result in off-target effects. The *lox* sites flanking the selectable marker easily prevented such an occurrence: after recombination by Cre, the *kan* cassette was removed from the genome, leaving only the small *loxP* site (Fig. 1D, E).

Strain characterization before HTS revealed that low arabinose concentrations extended the time required for cells to initiate exponential growth. One interpretation of this phenomenon may be related to the concentration of an essential protein required for bacterial growth. Although a cell is viable under low-expression conditions, it seems that accumulation of an essential gene product must reach some critical level for the cell to begin normal growth and division. At 13 μ M arabinose, the \downarrow *fabI* strain required 9 h to attain that level, whereas the \downarrow *murA* strain required 12 h (Fig. 2). Upon reaching this level, strains maintained normal growth rates unless expression was further reduced. Gene-specific factors such as the timing of induction and expression, translation rate and efficiency, and mRNA and protein stability likely play a role in the observed differences between strains.

It is precisely this result that permits the parallel screening strategy to be successful. Compared with the reference strain, an engineered strain in culture at some low arabinose concentration will require more time to reach saturating levels because of the limiting amounts of a single essential gene product. Should even a weak, but specific, inhibitor of that product be present in the culture medium, the number of active target molecules will be further reduced and result in a further growth delay. This lack of growth within a fixed time period (compared with the reference strain cultured under identical conditions with the same inhibitor) registers as a hit during HTS.

This can be demonstrated with known inhibitors and their targets^{28,29}. The \downarrow *murA* strain has increased sensitivity to the specific inhibitor fosfomycin (Table 2). Inhibitors of the later steps in the cell wall biosynthesis pathway (ampicillin and bacitracin) did not show increased activity against the \downarrow *murA* strain. This attests to the specificity and sensitivity of the screening approach.

HTS of these hypersusceptible strains identified new lead compounds that would not have been found through traditional cell-based screens (Fig. 3; Table 3). In parallel screening, compounds that inhibit the reference strain are temporarily excluded. At lower con-

centrations, these compounds can be rescreened against the strain array in an attempt to identify their mechanisms of action. Conversely, the same compounds can be tested against the strain array when each target is upregulated, scoring resistance as a hit. In fact, retesting the hits from the \downarrow *murA* screen at higher expression levels shows differences between or within the lead classes (Table 3). Confirmed by biochemical analysis (Table 3), these results suggest that preliminary structure–activity relationships may be defined that can help identify chemically attractive candidates early in the discovery process.

In addition to well-characterized essential gene targets, new targets identified through recent advances in genomics and bioinformatics can also be evaluated by this screening paradigm⁹. Essentiality testing of gene targets occurs simultaneously as a strain is engineered for HTS. Any newly defined essential target can then be screened for inhibitors, even in the absence of a defined biochemical activity (Table 1).

In summary, the advantages of using a strain array for antibacterial discovery are that (i) the strategy can be used for any target, including proteins that are difficult to assay *in vitro*, (ii) compounds identified in the screens will have antibacterial activity, (iii) the molecular target of an inhibitor of a hypersusceptible strain can be inferred or easily confirmed with a functional assay of the biochemical target, (iv) the screen for each different target is run with the same format and conditions, thereby streamlining the HTS process, (v) engineered strains are permanent tools that can be screened at any time and at low cost, and (vi) data collected from several screens of various targets may reveal compounds that inhibit more than one target simultaneously.

Experimental protocol

Reagents. Antibiotics were purchased from Sigma Aldrich (St. Louis, MO). For the purpose of duplicating experiments described in this paper, compounds 1–5 are available from Bristol-Myers Squibb Co. (New York, NY).

Bacterial strains and phage. *E. coli* DH5 α was used for plasmid manipulations. Strains N99, MG1655, and W3110 were used as sources of genomic DNA and as controls in phage plating experiments. *E. coli* DY329 (W3110 Δ *lacU169*, *nadA*:Tn10, *gal490*, λ *cl*₈₅₇ Δ [*cro-bio*]) was a gift of Don Court²⁴. It contains a defective λ prophage expressing the *gam*, *bet*, and *exo* recombination functions from the *cl*₈₅₇-regulated pL promoter. λ *cl*[–] and λ *vir* (gifts of Bob Weisberg) were used to test for the presence and absence of the defective prophage.

Plasmids. Essential target genes (together with their natural Shine–Dalgarno signals) were cloned for arabinose-regulated expression into the vector pBAD18 Cam^r using standard techniques³⁰. The gene for P1 Cre recombinase (along with its natural promoters, pR2 and pR3) was amplified from pRH104 (ref. 31) and cloned into a low-copy-number plasmid with a temperature-sensitive origin of replication (pJDP8).

Linear DNA substrate design and selection of recombinants. Construction of the *nadA* derivative of strain DY329 was accomplished through PCR of the *nadA* region on the chromosome of W3110 using the primers 5'-TCCTG-CACGACCCACCACTA-3' and 5'-CCGCTGCCCTATTGGTAT-3'. After purification, 100 ng of product was electroporated into *E. coli* strain DY329 in which expression of λ -*redgam* had been induced²⁴. Selection for *nadA* prototrophs and screening for sensitivity to tetracycline were carried out following standard methods³².

The λ prophage was removed by linear DNA transformation of *redgam*-induced cells with a 4.1 kb PCR product containing the phage λ attachment site, followed by selection at 42°C. Primer sequences for *attB* amplification were 5'-CGAACCGTAGGCCGATAAG-3' and 5'-GGCGAGGTGTCCAG-GTTG-3'. Temperature-resistant survivors were also tested for phage growth at 37°C (ref. 33) and for biotin prototrophy. The *ara*– and *pcnB*– derivatives of DY329 were constructed using a similar strategy.

Linear DNA substrates for deletion of target genes from the chromosome were synthesized by PCR using the *loxP*–kanamycin (*loxP*–*kan*) cassette from

pRH43 (ref. 16) as template. The following primers were used: N₅₀-TATCAC-GAGGCCCTTTCGTCTT-3' and 5'-N₅₀-TTTTCACCGTCATCACGAAAC-3', where "N" represents nucleotides composing the homology arms necessary for recombination. A 2.5 kb PCR product was generated from *loxP-kan* using primers with long tails (approximately 50 bp in length, represented by the curly lines in Figure 1D) that share sequence homology with the regions of the chromosome flanking a target gene (also represented by curly lines). This double-stranded DNA is used as a substrate for recombination in cells expressing λ -*redgam* via the homology tails. Kanamycin-resistant transformants are isolated in the presence of a plasmid containing the complementary copy of the target gene under arabinose control (Fig. 1B).

The kanamycin-resistant marker was removed by transformation with the temperature-sensitive Cre plasmid followed by selection on spectinomycin at 34°C. After incubation at 37°C, colonies were replica-plated on LB plates with and without kanamycin and grown at 37°C overnight. Kanamycin-sensitive colonies were screened by PCR to demonstrate loss of the marker (Fig. 1E). All chromosomal rearrangements were screened by PCR and confirmed by DNA sequencing (Fig. 1E). Primer details are available upon request.

Characterization of engineered strains. Growth analysis under different inducer concentrations was conducted in a Bioscreen C microbiology reader from Thermo Lab Systems (Helsinki, Finland). Cultures from each strain were grown overnight in medium containing various concentrations of arabinose. These served as seed cultures for testing growth in microtiter plates

under different conditions, such as inoculum size and inducer concentration. After monitoring these and other parameters, culture conditions under which the strains would become limiting for the essential gene target were determined. Before screening, mutant strains were also tested for sensitivity to antibiotics and drugs with known mechanisms of action to assess the target specificity of each strain. For such experiments, standard MIC determinations were done³⁴.

MurA biochemical assays. The activity of the MurA enzyme was measured by quantitating the UDP-GlcNAc-dependent release of inorganic phosphate from phosphoenolpyruvate according to published methods³⁵.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology website (<http://biotech.nature.com>) for details.

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