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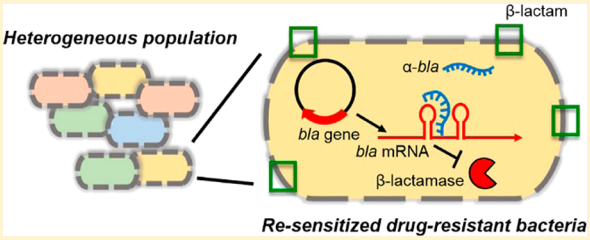
Sequence-Specific Peptide Nucleic Acid-Based Antisense Inhibitors of TEM-1 β-Lactamase and Mechanism of Adaptive Resistance

Colleen M. Courtney[†] and Anushree Chatterjee*[,†,‡]

[†]Department of Chemical and Biological Engineering and [‡]BioFrontiers Institute, 596 UCB, University of Colorado, Boulder, Colorado 80303, United States

Supporting Information

ABSTRACT: The recent surge of drug-resistant superbugs and shrinking antibiotic pipeline are serious challenges to global health. In particular, the emergence of β-lactamases has caused extensive resistance against the most frequently prescribed class of β-lactam antibiotics. Here, we develop novel synthetic peptide nucleic acid-based antisense inhibitors that target the start codon and ribosomal binding site of the TEM-1 β-lactamase transcript and act via translation inhibition mechanism. We show that these antisense inhibitors are capable of resensitizing drug-resistant *Escherichia coli* to β-lactam antibiotics exhibiting 10-fold reduction in the minimum inhibitory concentration (MIC). To study the mechanism of resistance, we adapted *E. coli* at MIC levels of the β-lactam/antisense inhibitor combination and observed a nonmutational, bet-hedging based adaptive antibiotic resistance response as evidenced by phenotypic heterogeneity as well as heterogeneous expression of key stress response genes. Our data show that both the development of new antimicrobials and an understanding of cellular response during the development of tolerance could aid in mitigating the impending antibiotic crisis.



KEYWORDS: *peptide nucleic acid, antibiotic resistance, β-lactamase, bet-hedging, adaptive resistance, β-lactamase inhibitor*

Antibiotic resistance is one of the world's most pressing health problems[1] with a number of antibiotic-resistant pathogens reported including multidrug-resistant New Delhi metallo-β-lactamase 1 producing *Klebsiella pneumoniae*,[2] carbapenem-resistant *Escherichia coli*,[3] and multidrug-resistant *Salmonella enterica*.[4] Although bacteria are rapidly developing resistance to current therapeutics, fewer therapeutics are being developed.[5,6] β-lactam antibiotics, including cephalosporins, penicillins, carbapenems, and monobactams, are some of the most frequently prescribed antibiotics for the treatment of bacterial infections; however, emergence of β-lactamases has caused extensive resistance against β-lactams.[7,8] Due to the onset of resistance from β-lactamases, β-lactam antibiotics are often combined with β-lactamase inhibitors, such as clavulanic acid, sulbactum, and tazobactam,[8−11] in therapeutic applications. Recently, resistance has also developed to the β-lactam/β-lactamase inhibitor combinations due to extended-spectrum β-lactamases and carbapenemases,[3] including New Delhi metallo-β-lactamase 1,[2] providing another avenue for widespread antibiotic resistance.

With an ever-shrinking arsenal of efficacious antibiotics, there is a need for developing novel antimicrobials. Sequence-specific antisense therapeutics have the ability to be pathogen-specific and offer a powerful antimicrobial strategy without nonspecific broad-spectrum activity.[12] Antisense therapeutics are nucleotide sequence based therapeutics that target specific RNA or DNA sequences and interact via complementary Watson−Crick base pairing between the target and antisense sequence,[13,14] thereby causing decrease in gene expression by blocking transcription, ribosomal binding, preventing ribosomal migration, or inducing cleavage by RNases.[15,16] Antisense therapies are not limited to natural nucleic acids,[17,18] but can also utilize synthetic nucleic acids such as locked nucleic acids (LNA), bicyclic nucleic acids (BNA), and peptide nucleic acids (PNA), among others.[13] These synthetic nucleic acids have been used to target a multitude of genes including LNA targeted against *ftsZ* in methicillin-resistant *Staphylococcus aureus*[19] and PNA targeted to *dnaK* in *Escherichia coli* and *Salmonella enterica*.[20] PNA, conjugated to cell penetrating peptides (CPPs) for increased transport into cells, has been used to target rRNA to disrupt protein synthesis and *acp*, to disrupt cell wall formation, in *E. coli*.[21] Although there are challenges associated with the delivery of antisense therapeutics,[13] the recent FDA approval of two antisense therapeutics, Fomivirsen for retinitis and Mipomersen for cholesterol reduction, highlights their potential use in practical applications.[17]

Despite the success of these methods, the ability of bacteria to adapt to antisense therapeutics has not been investigated in depth. Given that bacterial pathogens possess the intrinsic ability to acquire resistance via horizontal gene transfer[22,23] as well as develop adaptive antibiotic resistance,[6,24−26] there is a need to understand the mechanism of resistance to antisense therapeutics. Adaptive antibiotic resistance is the induction of resistance due to the presence of a specific signal or stressor and can be genetic[25,27] or nongenetic.[28] Nongenetic adaptive

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antibiotic resistance can be transient and is often observed as changes in gene expression.[28] Adaptive antibiotic resistance has been observed in the form of elevated efflux pump expression in *Acinetobacter baumannii*, in the presence of minocycline, ciprofloxacin, meropenem, tetracycline, and tigecycline,[29] and in *S. enterica*, in the presence of kanamycin.[30] It has also been observed as the up-regulation of genes in the anaerobic respiratory pathway in *Pseudomonas aeruginosa* in response to aminoglycoside exposure.[28]

Here we designed PNA-based antisense therapeutics to target the TEM-1 β-lactamase (*bla*) mRNA to resensitize drug-resistant *E. coli* (encoding TEM-1 β-lactamase) to β-lactam antibiotics and studied the mechanism of resistance to this antisense therapeutic strategy. We demonstrate that antisense inhibitors can serve as novel antibiotics and an alternative to conventional β-lactamase inhibitors, which have been typically developed to interfere with bacterial enzymes including transpeptidase, carboxypeptidase, and endopeptidase.[31] We performed a focused study to investigate the mechanism of resistance to the β-lactam/PNA-based antisense inhibitor combination. Through sequencing analysis of the antisense inhibitor target site, we find that mutants which develop tolerance to the β-lactam/antisense inhibitor combination do not have genetic mutations in the *bla* antisense inhibitor target site. Furthermore, we demonstrate that the mutants exhibit phenotypic heterogeneity as well as variable expression of representative stress response genes in the multiple antibiotic resistance regulon (Mar), general stress response, and SOS response pathways, implying a role of bet-hedging-based mechanism in the development of adaptive antibiotic resistance. This resistance mechanism has not been previously reported for nucleic acid targeting antisense therapeutics.

RESULTS AND DISCUSSION

Reversing β-Lactam Resistance by Targeting the Ribosomal Binding Site and Start Codon. We designed three novel antisense molecules, α-RBS, α-STC, and α-YUNR, against the ribosomal binding (RBS) site, translation start codon (STC), and a YUNR motif, respectively, proximal to the 5′ UTR of TEM-1 *bla* mRNA (Figure 1;Figure S1; Table 1) to prevent the production of truncated, but potentially active, β-lactamase enzyme.[32] *trans*-Antisense interactions have been shown to be effective at the RBS in preventing translation of CmeABC multidrug efflux pumps[33] and at the STC in targeting a plasmid-encoded Tn3 β-lactamase in *E. coli*.[32] Additionally, it has been shown that longer antisense sequences can reduce target translation by binding to both the RBS and STC such as the natural toxin/antitoxin system of *symE/symR*.[34] The antisense molecules studied here are predicted to sterically hinder the ribosome from binding and/or migrating on the *bla* transcript and consequently prevent its translation to β-lactamase protein.

To prevent degradation of antisense oligomers by endonucleases expressed by the host cell, we used non-natural antisense PNA oligomers. PNAs have a modified peptide backbone with nucleic acid functional groups and exhibit no known enzymatic cleavage, leading to increased stability in cells.[35] PNA molecules are known to have higher binding affinity and form more stable interactions with RNA and DNA than natural nucleic acids due to a neutral backbone.[14] TEM-1 β-lactamase has been targeted before with PNA molecules, but the molecules we present here are novel and have not been characterized before.[36]

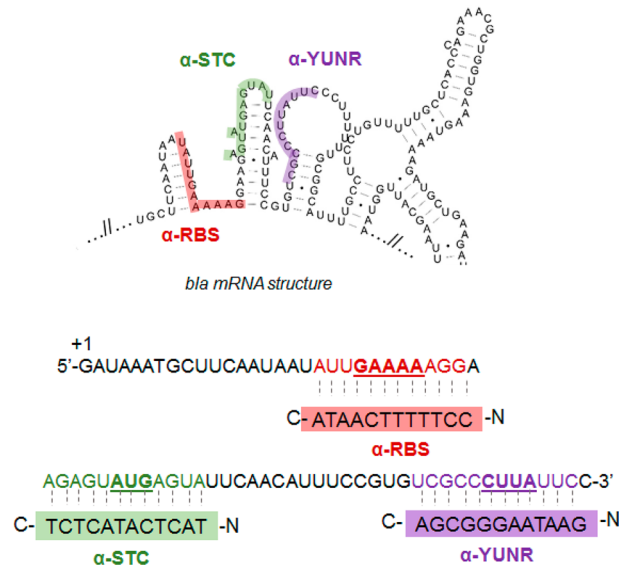

Figure 1. Design of antisense inhibitors against TEM-1 β-lactamase. Target sites of PNA-based antisense inhibitors are shown on the secondary structure of *bla* RNA encoding the TEM-1 β-lactamase enzyme. PNA molecules are 12-mers, where the N-terminus is conjugated to $(KFF)_3K$ cell penetrating peptide via an O-linker (Table 1). The ribosomal binding site (RBS), translational start site (STC), and YUNR motif are underlined in the *bla* sequence (bottom panel). Antisense agents α-RBS, α-STC, and α-YUNR targeting ribosomal binding site, translational start site, and YUNR motif, respectively, are shown.

Table 1. Peptide Nucleic Acid (PNA) Sequences Conjugated to O Linker and Cell Penetrating Peptide $(KFF)_3K$[a]

antisense molecule	conjugated components and PNA sequence
α-RBS	KFFKFFKFFK-O-cctttttcaata
α-STC	KFFKFFKFFK-O-tactcatactct
α-YUNR	KFFKFFKFFK-O-gaataagggcga

[a]The PNA sequences are written from the N-terminus to the C-terminus.

On the basis of previous stepwise target analysis with antisense PNA, the antisense oligomers were designed with the target sequence in the middle of the oligomer, with three to five nucleotides flanking the target region.[32] To prevent translation of β-lactamase, two 12-mer antisense oligomers, α-RBS (C-ATAACTTTTTCC-N; RBS underlined) and α-STC (C-TCTCATACTCAT-N; start codon underlined), were designed against the RBS and STC, respectively (Figure 1; Figure S1). Whereas α-RBS was designed to prevent the ribosomal binding, α-STC was designed to prevent ribosomal migration, both causing inhibition of translation of *bla* transcript. The third antisense inhibitor, α-YUNR (C-AGCGGGAATAAG-N; YUNR underlined), was designed to target the YUNR sequence motif on the stem loop between nucleotides 61 and 78 of the β-lactamase transcript (Figure 1; Figure S1). The YUNR motif (pyrimidine, uracil, any ribose nucleic acid, and a purine) has been shown to have high antisense binding affinity, due to the formation of intraloop hydrogen bonds facilitating a U-turn structure and is known to initiate rate-limiting interactions in a number of naturally occurring systems.[37,38] The targeted YUNR motif was in a single-stranded region of a stem loop proximal to the 5′ UTR in 14/19 free energy secondary structures of the *bla* RNA modeled using

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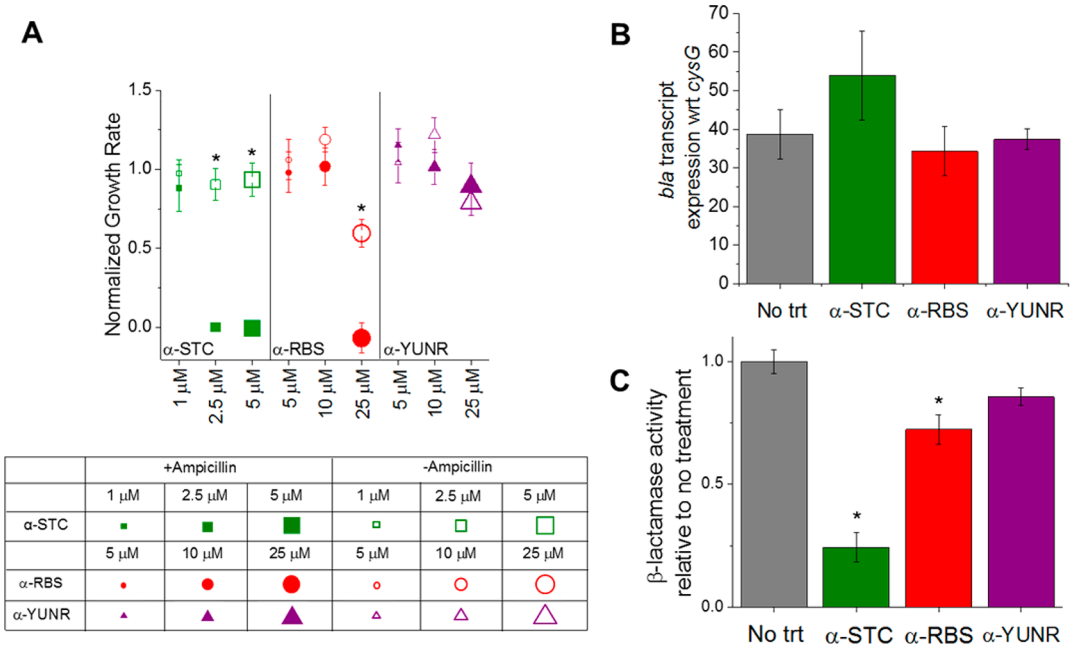

Figure 2. Minimum inhibitory concentration and mechanism of antisense inhibitors. (A) Normalized growth rate of overnight cultures after treatment with different concentrations of antisense inhibitors. Specific growth rate of cells under treatment with antisense inhibitors (y-axis) is normalized to specific growth rate in the absence of treatment. The cultures are treated with respective concentrations of antisense inhibitors either in the absence (open points) or in the presence of ampicillin (solid data points) (300 μg/mL). The MICs of α-STC, α-RBS, and α-YUNR are shown at 2.5, 25, and >25 μM, respectively. (B) *bla* mRNA expression in *E. coli* treated with respective antisense molecule in the absence of ampicillin. α-STC is at 5 μM, α-RBS is at 25 μM, and α-YUNR is at 25 μM for both (B) and (C). No significant change in *bla* mRNA expression is observed. (C) β-Lactamase activity assay with respective antisense molecule in the absence of ampicillin. Significant change is observed for α-STC and α-RBS. Data shown are an average of three independent experiments (error bars are standard deviation from average values). Significance ($p < 0.05$) is represented with an asterisk.

RNAstructure[39] (Figure S1). α-YUNR was also designed to prevent ribosomal migration, thus inhibiting translation of *bla* to β-lactamase enzyme.

The α-RBS, α-STC, and α-YUNR PNA molecules were designed as 12-mers for increased affinity to the target site.[40] The 12-mers were conjugated, via an O-linker, to $(KFF)_3K$ CPP for increased transport across the membrane into Gram-negative bacterial cells[40] (Table 1). The O-linker was added to reduce steric interference between the PNA and cell-penetrating peptide during target binding.[40] The 12-mer antisense sequences were searched against the *E. coli* K-12 genome (U00096.2) using NCBI BLAST to evaluate target selectivity and to avoid off-target interactions. α-RBS, α-STC, and α-YUNR searches returned no matches to the *E. coli* K-12 genome.[41]

We next evaluated the therapeutic potential of α-RBS, α-STC, and α-YUNR by identifying a minimum inhibitory concentration (MIC) at which the antisense inhibitors resensitized drug-resistant *E. coli* to 300 μg/mL ampicillin. The drug-resistant *E. coli* used for this study was Zymo Dh5a transformed with *bla* producing pAKgfp1 plasmid (Addgene plasmid 14076). α-RBS, α-STC, and α-YUNR were tested to identify a MIC between 1 and 25 μM based on concentrations reported in previous studies conducted in *E. coli* using CPP-conjugated PNA.[32,35,36,40] *E. coli* cultures treated overnight with respective antisense inhibitors, in the absence of ampicillin, grew similarly to untreated cells, demonstrating the nontoxic effect of the antisense inhibitors (Figure 2A; Figure S2A–C).

Strikingly, in the presence of 300 μg/mL ampicillin and 2.5 μM α-STC, the growth rate of ampicillin-resistant *E. coli* was significantly reduced ($p < 0.05$) (Figure 2A; Figure S2D). A similar inhibition of growth of drug-resistant *E. coli* was observed with the α-RBS antisense inhibitor at an elevated MIC of 25 μM α-RBS and 300 μg/mL ampicillin ($p < 0.05$) (Figure 2A; Figure S2E). In contrast, α-YUNR did not show growth inhibition up to 25 μM with 300 μg/mL ampicillin ($p > 0.05$) (Figure 2A; Figure S2F). Both α-STC and α-RBS resensitized drug-resistant *E. coli* to ampicillin and hindered cell growth, only in the presence of ampicillin, indicating gene-specific targeting of the *bla* gene.

The difference in MICs of α-STC and α-RBS could be attributed to the different binding affinities of α-STC and α-RBS to the respective target sites, leading to different extents of blocking ribosomal binding or migration.[32] Similarly, lower binding affinity could also explain the lack of *bla* translational inhibition by α-YUNR. The YUNR motif has not been studied previously as a target for PNA molecules and could be potentially ineffective due to the presence of tertiary structures that may sterically hinder the target site[13] or mismatch between the predicted YUNR stem loop and the native conformation in vivo. In addition, because YUNR stem loop motifs are important in natural rate-limiting antisense interactions, it is possible that the U-turn structure formed by the motif aids in binding only to other RNA stem loops and not to the structure void PNA molecules.

Translational Inhibition Mechanism of Action of α-RBS and α-STC Antisense Inhibitors. We next investigated the mechanism of action for α-STC, α-RBS, and α-YUNR antisense inhibitors. Using quantitative real-time polymerase chain reaction (qPCR), we measured expression levels of the *bla* gene in the presence of the antisense inhibitors with respect to uroporphyrin III C-methyltransferase, *cysG*, a moderately

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expressed housekeeping gene.[42] Studies were carried out at 5 μM α-STC, 25 μM α-RBS, or 25 μM α-YUNR in the absence of ampicillin. RNA expression analysis of *bla* transcript showed similar levels of *bla* RNA both in the absence and in the presence of treatment with the antisense inhibitors ($p > 0.05$) (Figure 2B), indicating that the antisense inhibitors did not inhibit the expression of *bla* transcript and were not causing significant degradation of transcripts. To evaluate the impact of the antisense inhibitors on translation of the *bla* gene, we used a β-lactamase activity assay to measure β-lactamase protein activity.[43] Indeed we observed that α-STC and α-RBS significantly reduced β-lactamase activity ($p < 0.05$) (Figure 2C). On the other hand, α-YUNR had no impact on protein activity ($p > 0.05$) (Figure 2C). These results demonstrate that α-STC and α-RBS reduced β-lactamase activity, but did not affect *bla* transcript levels, thus indicating that α-STC and α-RBS act via a translational inhibition mechanism. These results are consistent with the growth behavior shown in Figure 2A and Figure S2, where α-STC and α-RBS affect the growth of drug-resistant *E. coli* in the presence of ampicillin and α-YUNR had no impact on cell growth.

α-STC Antisense Inhibitor Restores Ampicillin Sensitivity. To evaluate the therapeutic potential of antisense inhibitors, we investigated the best-performing antisense inhibitor, α-STC, in the following studies. Overnight cultures of ampicillin-resistant *E. coli* were pretreated with different concentrations of α-STC, followed by treatment with ampicillin. Because α-STC inhibits β-lactamase production, we expected that α-STC would restore the bactericidal effect of ampicillin. Indeed, α-STC decreased cell viability, at the MIC of 2.5 μM and higher, by at least 1000-fold within the first 3 h of treatment with ampicillin (Figure 3A). Below the MIC of α-STC (no treatment case and 1 μM α-STC), colony-forming units (CFU) increased with time (Figure 3A).

We next evaluated the degree of resensitization exerted by α-STC in the presence of various concentrations of ampicillin, above and below the MIC determined for α-STC. Three concentrations of α-STC were tested: no treatment, 0.5 μM α-STC (5-fold below MIC), and 5 μM α-STC (2-fold above MIC) (see Methods) (Figure 3B). In the absence of treatment, ampicillin-resistant *E. coli* was able to grow up to 300 μg/mL ampicillin without inhibition and showed a gradual decrease in growth until 700 μg/mL ampicillin, where no growth was observed. Below the MIC level of 0.5 μM α-STC ampicillin-resistant *E. coli* grew unhindered up to a reduced ampicillin concentration of 250 μg/mL and showed no growth at 550 μg/mL when compared to the no treatment case. Strikingly, at 5 μM α-STC (above the MIC), ampicillin-resistant *E. coli* only grew unhindered without ampicillin and showed a decrease in growth as low as 25 μg/mL and no growth at 75 μg/mL ampicillin, elucidating a drastic 10-fold decrease in the MIC of ampicillin compared to the nontreated cultures, exhibiting behavior closer to antibiotic-sensitive parent *E. coli* strain (Figure S3). Data fitting (see Methods) of the drug-sensitivity curves showed that the slope of the transition state, from resistant to sensitive, is altered drastically and depends on both the concentration of the α-STC antisense inhibitor and ampicillin (Figure 3B). A >2-fold increase in negative slope above the MIC (−0.010 OD/μg/mL) compared to below the MIC (−0.0038 OD mL μg^{-1}) and no treatment (−0.0051 OD mL μg^{-1}) indicates that whereas the α-STC is resensitizing the *E. coli* to ampicillin, it is changing the sensitivity landscape with respect to the resistant strain.

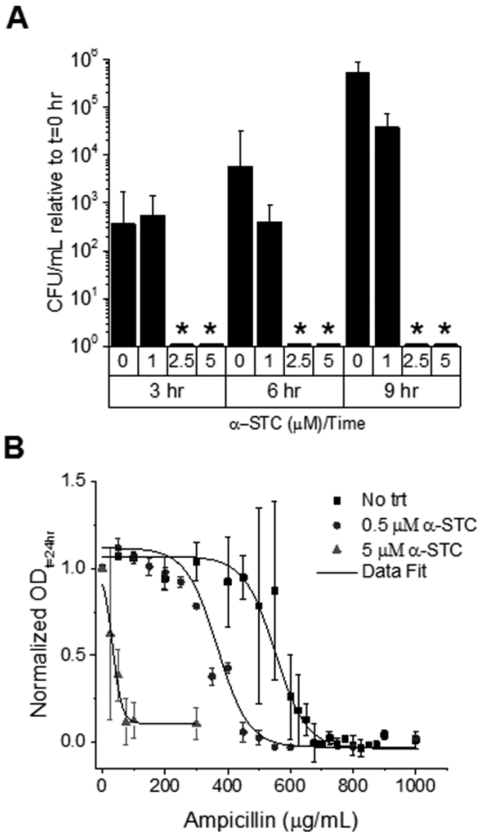

Figure 3. Resensitization of drug-resistant bacteria using α-STC. (A) Colony-forming units per milliliter (CFU/mL) for cultures treated with different concentrations of α-STC and 300 μg/mL ampicillin. Cultures with significant decrease in growth ($p < 0.05$) are represented with an asterisk. (B) Ampicillin sensitivity analysis for cultures treated with 0, 0.5 (below MIC), and 5 μM (above MIC) of α-STC and a range of ampicillin concentrations. Optical density (OD) of cultures treated with a range of α-STC and ampicillin concentrations for 24 h is shown. The data are normalized to the OD at 24 h with 0 μg/mL ampicillin. The three conditions demonstrate different degrees of sensitivity as shown using a Boltzman data fit. Data shown are an average of three independent experiments (error bars are standard deviation from average values).

Evaluating the Emergence of Tolerance to Ampicillin/α-STC Antisense Inhibitor Combination at the MIC Demonstrates Heterogeneity in Growth Rate and MIC of Ampicillin. Because resistance has been reported for enzyme-based β-lactamase inhibitors,[9] we investigated the potential emergence of tolerance to the α-STC/ampicillin combination both above and at the MIC. Ampicillin-resistant cultures were pretreated overnight with either 5 μM (above MIC) or 2.5 μM (at MIC) α-STC and then subjected to selection pressure of 300 μg/mL of ampicillin and 5 or 2.5 μM α-STC, respectively, for 24 h. Interestingly, we did not observe the emergence of tolerance when ampicillin-resistant cultures were treated with 5 μM α-STC and 300 μg/mL of ampicillin. However, when we subjected the ampicillin-resistant cultures to MIC concentrations of α-STC (2.5 μM), 2 of 35 cultures broke the trend and showed emergence of tolerance. The two cultures that developed tolerance to ampicillin/α-STC combination over 24 h were collected and are here referred to as mutant populations 1 and 2 (Figure 4A; Figure S4A). Mutant populations 1 and 2 were diluted and regrown under selection pressure for another 24 h. Notably, the mutants grew (Figure

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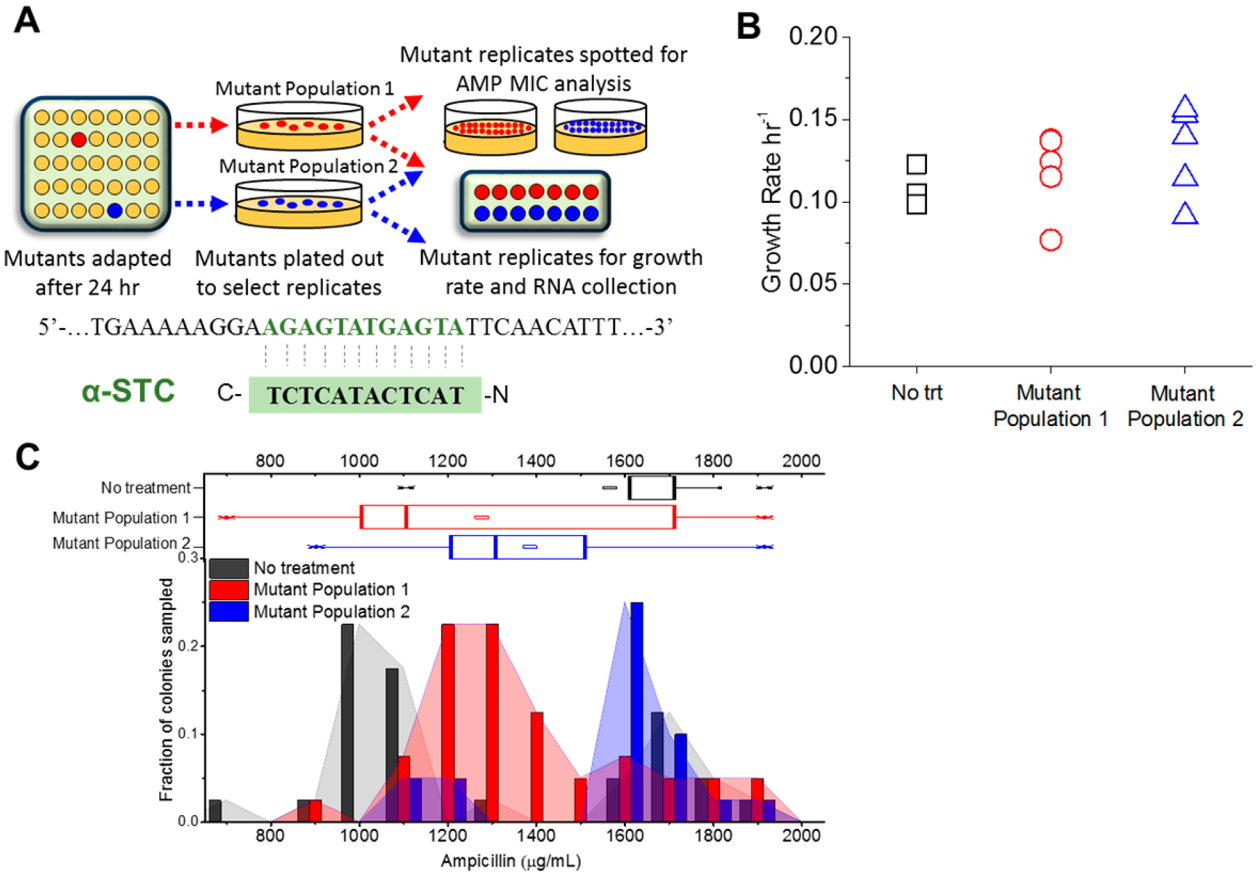

Figure 4. Mutants adapted to antisense inhibitors demonstrate heterogeneity in growth rate and MIC of ampicillin. (A) Schematic showing the process of selecting adapted mutants. Thirty-five independent cultures were grown at the MIC level of 2.5 μM α-STC and 300 μg/mL ampicillin to investigate the emergence of resistance. The two mutant populations were plated onto solid media, and individual colonies were sampled from each population. Sequencing of the *bla* gene in individual colonies confirmed that the target site of α-STC was not mutated in the nontreated population (n = 3), mutant 1 population (n = 3), and mutant 2 population (n = 3). (B) Growth rate for individual biological replicates in nontreated population (n = 6), mutant population 1 (n = 6), and mutant population 2 (n = 5). (C) MIC of ampicillin for individual replicates from nontreated population (n = 40), mutation 1 population (n = 40), and mutant 2 population (n = 40) was measured ranging from 0 to 2000 μg/mL of ampicillin (lower panel). The box plot of the data (upper panel) analyzes only those replicates for which the MIC was within the ampicillin range sampled (0–2000 μg/mL). The box plot shows the range of the data in asterisks, the vertical line in the box represents the median, the small box represents the mean, and the horizontal capped lines represent the lower (25%) and upper (75%) quartiles.

4B; Figure S4B), confirming they were stable mutants that had developed tolerance to α-STC and their growth was not an artifact of ampicillin or α-STC degradation.

The *bla* gene in the mutant populations was sequenced to determine whether the cause of tolerance was a mutation in α-STC's target site. Three biological replicates each from mutant population 1, mutant population 2, and a nontreated population were grown overnight, after which the plasmid containing the *bla* gene was extracted and the *bla* gene was sequenced (see Methods). Interestingly, no genetic mutations were found in the α-STC target site (Figure 4A; Table S1). In contrast to our findings, piperacillin/tazobactam, a traditional β-lactam/β-lactamase inhibitor drug combination, has been shown to lose clinical efficacy in part due to the development of mutations at the target site of tazobactam in the β-lactamase gene.[11]

Interestingly, the biological mutant replicates showed high variability in growth rate compared to the nontreated biological replicates (Figure 4B; Figure S5). The heterogeneity in growth rate led us to investigate the MIC of ampicillin as a second indicator of heterogeneity. We analyzed the MIC of ampicillin, ranging between 0 and 2000 μg/mL, for 40 individual colonies from each population to investigate heterogeneity in ampicillin sensitivity. For the colonies sampled, 20 of the 40 from the nontreated population, 29 of the 40 from mutant population 1, and 38 of the 40 from mutant population 2 showed MICs between 0 and 2000 μg/mL. For the following analysis only colonies with a MIC in the range sampled were considered. Averages of the MIC of ampicillin for the populations were 1555 μg/mL for the nontreated population, 1269 μg/mL for mutant population 1, and 1376 μg/mL for mutant population 2 (Figure 4C; Figure S6). The analysis of population averages elucidated that the average MIC in the nontreated population was significantly different from that of mutant populations 1 (p = 0.002) and 2 (p = 0.007). Furthermore, we observed a wider range of MIC of ampicillin for mutant population 1 (700–1900) μg/mL and mutant population 2 (900–1900 μg/mL) compared to the nontreated population (1200–1900 μg/mL) (Figure 4C), indicating greater heterogeneity in the mutant populations. This is also evident from box plots that show larger inner (25%) and upper (75%) quartiles of the MICs in mutant populations 1 and 2, whereas the nontreated population MICs are centered on the mean, with tight inner and upper quartile ranges (Figure 4C; Figure S6B). This difference in the

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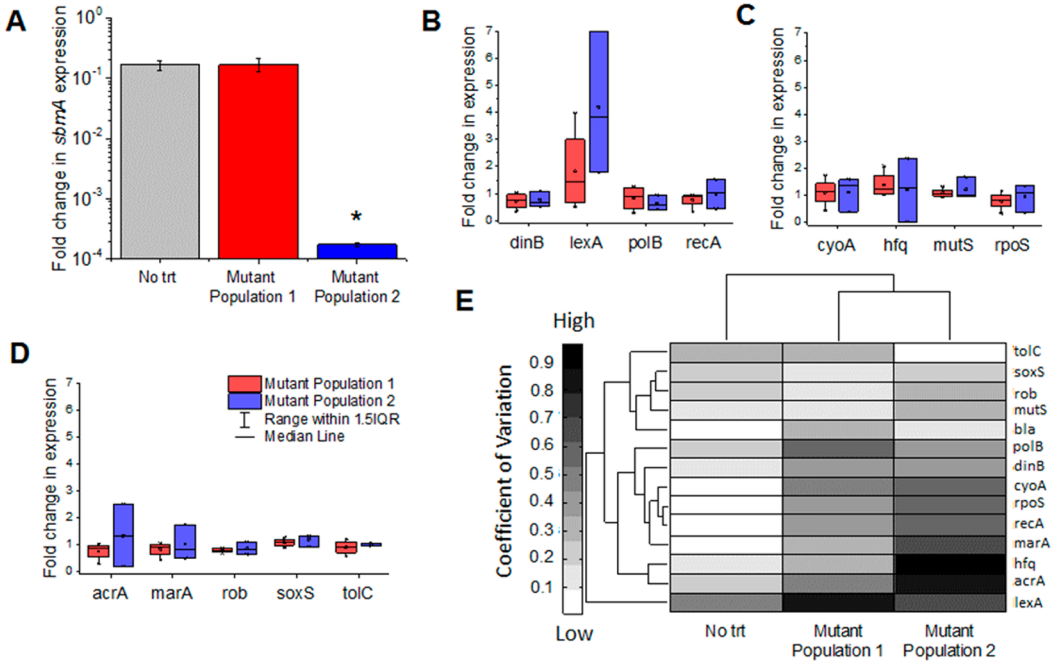

Figure 5. Mutants adapted to antisense inhibitors demonstrate gene expression heterogeneity. (A) Expression of *sbmA* with respect to moderately expressed housekeeping gene *cysG*. Significance ($p < 0.05$) is represented with an asterisk. (B–D) Expression of genes in the SOS response pathway (B), general stress response pathway (C), and Mar regulon (D) with respect to *cysG* in the nontreated population ($n = 3$) and mutant populations 1 ($n = 4$) and 2 ($n = 3$). (E) Hierarchical clustering analysis showing significant difference in the coefficient of variation between nontreated case and mutant populations 1 and 2. Heatmap values indicate coefficient of variation of $-\Delta C_q$ between biological replicates of nontreated population ($n = 3$), mutant population 1 ($n = 4$), and mutant population 2 ($n = 3$). Clustering is based on Euclidean distance. Data shown are an average of *n* biological replicates (error bars are standard deviation from average values).

spread of the data shows that the MIC of ampicillin for biological replicates from the mutant populations is more heterogeneous than that of the nontreated population.

Similar phenotypic heterogeneity has also been observed in *S. enterica* for adaptive resistance to bile salt sodium deoxycholate and has been described as an indicator of a bet-hedging phenomenon.[44] Bet-hedging is an evolutionary principle in which cells in a population vary gene expression to find an adaptive state by stochastic chance.[45] The nature of bet-hedging and stochastic variation generates heterogeneity within a population. Recently, it was shown that resistance of *S. enterica* to kanamycin is facilitated by bet-hedging observed as heterogeneous expression of *ompC* efflux porin, which generates two populations, one that is resistant to kanamycin with low *ompC* and one that is not resistant to kanamycin with high *ompC*.[30] It was also shown that virulence in *S. enterica* is facilitated by the development of two populations, a virulent and an avirulent population, through a bet-hedging-based phenomenon.[46] Interestingly, we identified two populations within mutant population 1 based on the MIC of ampicillin, one that is centered at 1000 μg/mL ampicillin and one centered at 1700 μg/mL ampicillin. The observed heterogeneity in growth rate and development of two populations in MIC of ampicillin in mutant population 1 led us to perform gene expression studies to further investigate whether bet-hedging and heterogeneity would be observed in *bla* resistance and stress response gene expression as an adaptive antibiotic resistance mechanism.

Adapted Populations Exhibit Differential *sbmA* and Heterogeneous Stress Response Gene Expression. We next measured the change in expression in a previously reported PNA efflux protein, *sbmA*, and a set of key stress response genes to elucidate a tolerance mechanism. The *sbmA* gene is a peptide transporter that was previously shown to be an importer of PNA.[47] We measured the expression level of *sbmA* in the nontreated population as well as in mutant populations 1 and 2. Mutant population 2 had a significant decrease in expression of *sbmA* relative to both the nontreated population and mutant population 1 ($p < 0.05$) (Figure 5A). Mutant population 1 did not exhibit differential expression of *sbmA* relative to the no treatment population ($p > 0.05$). This finding confirms that mutant populations 1 and 2 are distinct mutants that adapted by unique mechanisms to the α-STC/ampicillin combination and further supports our hypothesis of bet-hedging as a mechanism of resistance. The decreased expression of *sbmA* in mutant population 2 agrees with previously reported resistance to PNA observed as a mutation in the upstream region of the *sbmA* gene, which reduced its expression.[47]

To further probe the tolerance mechanism, the expression of 13 representative stress response genes was measured using qPCR (Figure 5B–D). Stress response genes were chosen on the basis of a survey of common drug resistance, global stress response, and mutagenesis genes. We examined *marA*, a transcriptional activator of broad range efflux pumps,[48,49] and two coupled efflux genes that *marA* activates, *acrA* and *tolC*.[49] *acrA* and *tolC* are outer membrane proteins implicated in multidrug efflux.[48,50] The expression level of *rpoS*, a sigma factor for global stress response and stationary phase, was examined because it is shown to regulate 10% of the *E. coli* genome during stress and is associated with DNA damage repair.[51,52] Expression of cytochrome oxidase subunit, *cyoA*,[53] and *hfq*, a regulator of sRNA interactions,[54] was examined for their role in stress response upstream of *rpoS*.[55] Expression of

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two error-prone DNA polymerases associated with stress response was measured, *dinB*, for DNA polymerase IV that does not exhibit proofreading[56] and *polB*, encoding DNA polymerase II, which exhibits proofreading.[25] Expression of *mutS*, a gene encoding a mismatch repair enzyme, was examined because it was shown to reduce mutagenesis in *E. coli* during antibiotic stress.[57] The expression of three transcriptional regulators of stress response, *lexA*, a transcriptional repressor of SOS stress response,[58] *rob*, which codes for a transcriptional activator of stress response genes,[48] and *soxS*, which regulates the expression of >100 genes during stress response,[59] was measured. In addition, *recA* expression was measured for its role in homologous recombination and in initiating the SOS response by acting as a protease that cleaves the *lexA* stress response repressor.[60,61]

Strikingly, the fold change in expression level for the set of stress response genes relative to the nontreated case ($2^{-\Delta\Delta C_q}$) exhibited a large range of fold changes across biological replicates (Figure 5B–D; Figure S7A). Some noteworthy genes with large relative fold change range included *hfq*, which varied from 0.03 to 1.7 (Figure 5C), *acrA*, which varied from 0.18 to 2.5 (Figure 5D), and *lexA*, which varied from 0.5 to 7 (Figure 5B). Interestingly, *bla* mRNA was expressed tightly across all biological replicates, with a fold change ranging between 0.8 and 1.7 for the mutant populations with respect to the nontreated case (Figure S7B). The observation of large deviations in fold change for individual mutant biological replicates and the observed phenotypic heterogeneity in growth rate and MIC of ampicillin within mutant populations led us to analyze the variance within the mutant population's gene expression.

Notably, we observed significant gene expression heterogeneity across the seven biological replicates from mutant populations 1 and 2 (Figure 5B–D). The three populations, no treatment, mutant population 1, and mutant population 2, were clustered (see Methods) to examine the relationships between the coefficients of variation (COV) of cycle numbers (C_q) of the stress response genes measured using qPCR (Figure 5E) with respect to housekeeping gene *cysG* (ΔC_q) (Figure S7C). Hierarchical clustering analysis showed that the mutant populations were clustered separately from the nontreated case based on the high degree of variability. Higher COVs were observed in 10 of 13 stress response genes within mutant population 1 and in 12 of 13 stress response genes in mutant population 2 (Figure 5E). The existence of a few tightly expressed genes indicated that the variation seen in specific stress response genes is unique to the adapted state.

Surprisingly, mutant populations 1 and 2 both showed at least a 30-fold increase in the COV for *marA* expression compared to nontreated case, suggesting that varied levels of transcriptional activation of broad range efflux pumps may be important in obtaining tolerance to α-STC. MarA has been identified as an activator of efflux pumps that gives resistance to unrelated antibiotics, classifying it as an activator of a nonspecific efflux system.[62] Surprisingly, variance in two efflux pump genes regulated by *marA*, *acrA* and *tolC*, did not follow the same trend where *acrA* had greater COV in mutant populations 1 and 2 and *tolC* expression had similar or reduced COV in the mutant populations. Interestingly, the MtrCDE efflux system in *Neisseria gonorrheae* has been shown to confer resistance to a diverse range of antimicrobial peptides and other hydrophobic agents[63,64] and is stated to be similar to the *acrA* locus in *E. coli*, which had increased variance in the mutant populations.

Both mutant populations displayed high COV for *rpoS* expression, which has been implicated in facilitating cross-protection[54] and shown to be key in obtaining antibiotic resistance to biapenem and imipenem in *P. aeruginosa*.[65] Accordingly, *cyoA* and *hfq*, upstream regulators of *rpoS*, showed increased COV in mutant populations. Interestingly, the expression level of *recA* and *lexA* was varied in mutant populations. When RecA cleaves the LexA repressor, a number of SOS-related genes are derepressed, thus initiating a global stress response as reflected in the increased variance of two SOS-related genes, *dinB* and *polB*. Within the mutant populations, *mutS*, which has previously been shown to reduce lethality of β-lactam antibiotics,[57] had increased variance, but it is unclear whether this is in response to α-STC or the β-lactam in the therapeutic combination. Stress response gene expression signatures of resistance to traditional β-lactamase inhibitors has not been reported previously, so we could not ascertain whether there is similarity in resistance signatures to the designed synthetic β-lactamase antisense inhibitor.

The heterogeneity observed in growth rate and MIC of ampicillin, the difference in *sbmA* expression between mutant populations 1 and 2, and expression heterogeneity of stress response genes indicate a role of evolutionary bet-hedging in the development of adaptive antibiotic resistance to the ampicillin/α-STC combination. Preventing bet-hedging is not currently understood, but limiting the bet-hedging of stress response genes could potentially reduce the fitness of bacteria to adapt to antibiotics. Antisense therapeutics have the potential to target any gene in the genome. This can allow for a limiting of bet-hedging using a combination antisense therapy of molecules targeted not only to the essential or antibiotic resistance conferring gene but also to stress response genes such as *marA*, which had highly variable expression in the mutant populations. Studying the genes that enable and take part in bet-hedging will allow for a better understanding of how to produce efficacious antibiotics.

Whereas α-STC and α-RBS were successful at resensitizing resistant bacteria, they also demonstrate a clear opportunity for future antisense-based antimicrobials. Antisense molecules could be designed to target other resistance mechanisms such as NDM-1 β-lactamase, carbapenemase, extended spectrum β-lactamase, aminoglycoside acetyltransferase, and dihydropteroate synthase.[66,67] Antisense-based therapeutics are inherently specific due to their sequence-based targeting. This makes them advantageous as antibiotics because it removes side effects associated with broad-range antibiotics including preventing extreme changes in the resistome and populations of the patients microbiome.[68] We show that antisense inhibitors may provide an opportunity for mitigating the first sign of emergence of antibiotic resistance with quick development of antisense-based inhibitors, which require only the sequence of an identified target site and synthesis of the cognate antisense molecule. In the development of new antibiotics and resistance inhibitors it is important to study the emergence of resistance, both in vitro and in vivo, to begin mitigating the antibiotic resistance crisis. Targeting resistance head-on rather than waiting for it to develop could be useful in designing antibiotics that prevent resistance and remain efficacious for years to come.

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METHODS

Bacterial Strains and Cell Culture Conditions. pAKgfp1 plasmid was a gift from Attila Karsi (Addgene plasmid 14076) encoding TEM-1 β-lactamase gene, *bla*.[69] The plasmid was cloned into chemically competent Zymo DH5a *E. coli* (Expressys). Liquid cultures were grown in 2% LB, incubated at 37 °C, and shaken at 225 rpm. Solid cultures were grown on 2% LB broth, 1.5% agar at 37 °C. Ampicillin sodium salt (Sigma-Aldrich) was used for selection. Optical density measurements were taken using a Tecan GENios at 562 nm with a bandwidth of 35 nm. Growth rates were calculated from the exponential growth phase of the growth curves (Figure S2). MICs were determined by observing the lowest concentration at which the antisense inhibitor and/or ampicillin prevented a measurable increase in optical density or observable colonies on solid media over 24 h. All bacterial freezer stocks were stored in 40% glycerol at −80 °C.

Colony-Forming Unit Analysis. Cultures were sampled at respective time points, and serial dilutions were performed ranging from 10^2 to 10^{10}. Dilutions were plated on solid media and 300 μg/mL ampicillin sodium salt and grown at 37 °C for 24 h followed by cell counting. CFU per milliliter was normalized to $t = 0$ for each respective condition. Raw data can be seen in Figure S8.

Antisense Inhibitors. PNAs were purchased from PNA Bio, Inc. (Thousand Oaks, CA, USA). PNA was resuspended in 5% DMSO in water at 100 μM. Working stocks were stored at 4 °C and long-term stocks at −20 °C to limit freeze−thaw cycles.

***bla* RNA Collection.** Cultures were pretreated overnight in respective PNA in liquid media in the absence of ampicillin and collected for RNA at 16 h. Three biological replicates were used for *bla* mRNA expression analysis.

Mutant Biological Replicate Collection. Biological replicates from each mutant population were used for sequencing and gene expression analysis. Mutant populations 1 and 2 were regrown from respective freezer stocks in liquid media with 2.5 μM α-STC and 300 μg/mL ampicillin at 37 °C with shaking. At 16 h, 1:100 dilutions were plated onto solid media with 300 μg/mL ampicillin and grown at 37 °C for 16 h. Individual colonies were selected and regrown in liquid media, 2.5 μM α-STC, and 300 μg/mL ampicillin. For samples used in gene expression analysis, samples were collected when they reached mid log phase (OD 0.4−0.5). This method was used to sample individual biological replicates in the mutant populations.

Measuring the MIC of Ampicillin within Mutant Isolates. Freezer stocks of a nontreated population and mutant populations 1 and 2 were plated onto solid media with a selection pressure of 300 μg/mL of ampicillin. Individual colonies were selected from the nontreated population (n = 40), mutant 1 population (n = 40), and mutant population 2 (n = 40) and suspended in 100 μL of liquid medium. Five microliters of each colony suspension was spotted onto solid medium with ampicillin concentrations ranging between 0 and 2000 μg/mL. After 24 h of growth at 37 °C, colonies were examined to determine their MIC. The MIC was reported as the concentration at which no cell growth occurred. Analysis of populations and box plot statistics was performed using OriginPro 9.1. Raw data for isolates are shown in Figure S6A.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction. Fifty microliters of the respective culture was added to Bacteria RNAprotect (Qiagen) and pelleted for storage following the manufacturer's instructions. Samples were flash frozen in an ethanol dry ice bath and stored at −80 °C. Precautions were taken to protect RNA from RNases using RNaseZap (Life Technologies). RNA was extracted from frozen cell pellets using a GeneJET RNA purification kit (Thermo Scientific) followed by treatment with Turbo DNA-*free* (Ambion). Fifty nanograms of cDNA was synthesized using Maxima Universal First Stand cDNA synthesis kit (Thermo Scientific). Primers for qPCR were purchased from Integrated DNA Technologies and are listed in Table S2. One and a half nanograms of cDNA was used for qPCR with Maxima SYBR Green qPCR master mix with ROX normalization (Thermo Scientific) using Illumina Eco qPCR system. Transcript levels were analyzed using the ΔC_q method with respect to moderately expressed housekeeping gene *cysG*.[42] Transcript levels were further analyzed for the mutant populations using the $\Delta\Delta C_q$ method with respect to the nontreated populations.

***β*-Lactamase Activity.** Fluorocillin Green 495/525 β-lactamase substrate soluble product (Life Technologies) was used at a concentration of 2.2 μM as a β-lactamase substrate and measured using a Tecan GENios microplate reader in black flat-bottom 96-well plates at 485/535 nm with a bandwidth of 35 nm. Three biological replicates were grown from colonies for 12 h in liquid medium in the presence of the respective antisense inhibitors, diluted 1:10 into liquid medium with Fluorocillin Green, and monitored in the Tecan GENios for 5 h at 37 °C measuring every 2 min. The slope of the exponential, linear region of fluorescence measured was used as a measure of β-lactamase activity as described in Kong et al.[70]

Ampicillin Sensitivity Analysis. Three biological replicates were selected from colonies and pretreated for 16 h with respective α-STC concentration in liquid media, followed by 1:100,000 dilution into liquid medium with respective concentration of ampicillin and α-STC and allowed to grow with shaking at 37 °C for 24 h. The final OD at 562 nm at 24 h was used for data analysis. Data fitting analysis of sensitivity curves was performed in Origin Pro 6.1. Data were fit to a sigmoidal/decay Boltzman function.

Sequencing of *bla* Gene on Plasmid. Mutant cultures were regrown from freezer stocks with three biological replicates being sampled from each mutant population 1 and mutant population 2 as well as the nontreated control. The mutant cultures were regrown in 300 μg/mL ampicillin for 16 h, and the plasmid was extracted using a GeneJET Plasmid Miniprep Kit (Thermo Scientific). The samples were sequenced using Sanger sequencing (Genewiz) and primer 5′-GAATTCGAATTC**TCAGAAGTAAGTTGGCCGCA**-3′. The sequence homologous to the *bla* gene is in bold.

Data Analysis. Data are represented as the mean ± standard deviation. Single-factor ANOVA was performed with confidence of $p < 0.05$. Replicates shown are biological replicates.

Clustering Analysis. The COV is defined as the standard deviation divided by the mean of the samples. The COV was calculated for the nontreated population (n = 3), mutant population 1 (n = 4), and mutant population 2 (n = 3) using data from the ΔC_q method with respect to *cysG*. The clustergram function in the MATLAB Bioinformatics Toolbox (The Mathworks, Inc., Natick, MA, USA) was used to perform hierarchical clustering of the COVs for gene expression analysis and to generate the heatmap and dendrogram. The standard

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setting of optimal leaf ordering, Euclidean pairwise distance calculation, and an unweighted average distance linkage function were used for the clustergram function.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00042.

Supplementary tables, figures, and references (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*(A.C.) Mail: 3415 Colorado Avenue, 596 UCB, University of Colorado, Boulder, CO 80303, USA. E-mail: chatterjee@colorado.edu. Phone: (303) 735-6586. Fax: (303) 492-8425.

Notes

The authors declare no competing financial interest.

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