Selective Advantage of Resistant Strains at Trace Levels of Antibiotics: a Simple and Ultrasensitive Color Test for Detection of Antibiotics and Genotoxic Agents

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Received 26 August 2010/Returned for modification 9 November 2010/Accepted 25 December 2010

Many studies have examined the evolution of bacterial mutants that are resistant to specific antibiotics, and many of these focus on concentrations at and above the MIC. Here we ask for the minimum concentration at which existing resistant mutants can outgrow sensitive wild-type strains in competition experiments at antibiotic levels significantly below the MIC, and we define a minimum selective concentration (MSC) in Escherichia coli for two antibiotics, which is near 1/5 of the MIC for ciprofloxacin and 1/20 of the MIC for tetracycline. Because of the prevalence of resistant mutants already in the human microbiome, allowable levels of antibiotics to which we are exposed should be below the MSC. Since this concentration often corresponds to low or trace levels of antibiotics, it is helpful to have simple tests to detect such trace levels. We describe a simple ultrasensitive test for detecting the presence of antibiotics and genotoxic agents. The test is based on the use of chromogenic proteins as color markers and the use of single and multiple mutants of Escherichia coli that have greatly increased sensitivity to either a wide range of antibiotics or specific antibiotics, antibiotic families, and genotoxic agents. This test can detect ciprofloxacin at 1/75 of the MIC.

The increase in drug-resistant bacteria is a major health concern, as multidrug-resistant infections have reached an alarming point (2, 17, 30). One contributor to this rise in resistant bacteria is the prevalence of antibiotics in household and farm products, as antibiotics such as fluoroquinolones are used to treat farm animals and to increase farming output (23). Much work has focused on the evolution of antibiotic resistance, including the occurrence and proliferation of mutants at different concentrations of antibiotics (e.g., see references 19 and 35). Drlica and coworkers (18, 19) have defined a mutant selection window (MSW) of concentrations at which resistant microorganisms are preferentially selected, beginning at or near the MIC and continuing up to a point where mutants cannot grow, the mutant prevention concentration (MPC). However, recent studies have shown that resistant mutants already are prevalent in the human microbiome. For instance, Sommer et al. (44) tested culturable bacteria obtained from the intestinal microflora of two individuals. On average, bacteria from individuals 1 and 2 were resistant to 9 and 5 antibiotics, respectively, of the 13 antibiotics tested. A total of 70% of the bacterial species from one individual were resistant to 10 of the 13 antibiotics tested. Continual exposure to trace amounts of antibiotics may be responsible. Cells certainly recognize such levels, as Davies and coworkers have shown that very low levels of antibiotics have signaling effects on cells, stimulating transcription from a multitude of operons (24, 51, 52). It is therefore relevant to ask, for each antibiotic, how low a concentration will still select for existing resistant mutants in competition experiments. For each antibiotic and each microorganism, we might define such a concentration in terms of a fraction of the MIC and label it the minimum selective concentration (MSC). We show here that for the two antibiotics studied, ciprofloxacin (Cip) and tetracycline (Tet), the MSC is significantly below the MIC in Escherichia coli.

The development of tests to detect residual levels of antibiotics in milk, eggs, farm soil, waste, and water is important for monitoring the levels of these compounds to which populations are exposed. For example, such tests have revealed the presence of levels of 0.7 to 124.5 ng/ml of ciprofloxacin in hospital wastewater (25). Milk is constantly screened, as the European Union requires the combined levels of two fluoroquinolones (enrofloxacin plus ciprofloxacin) in milk to be no greater than 100 ng/ml (see reference 7).

There are several types of assays for residual antibiotics, including physical chemical tests, immunological screens, biological tests, and in some cases, combinations of these. Some of these physical chemical tests, such as high-pressure liquid chromatography (HPLC) (e.g., see reference 14), liquid chromatography coupled to mass spectroscopy (14), and surface plasmon resonance (49), can detect several nanograms/milliliter of fluoroquinolones but require elaborate instrumentation and are less useful for initial determinations in rural areas or developing countries. Immunological methods can achieve a sim-
ular resolution (e.g., Charm MRL [maximum permissible residue level]) but are specific for each antibiotic class and also are expensive. Combinations of the two methods are being developed for detailed analysis, such as an alternating current impedance method combined with an immunosensor for ciprofloxacin (47). Biological tests have the advantage of often requiring much simpler methodology. Conventional tests depending on direct demonstration of growth inhibition, such as those with discs placed on a petri dish, are now being superseded by tests using different types of biosensors. A strain of *Escherichia coli* has been described that can detect low levels of tetracyclines by using an engineered strain with the luciferase enzyme under the control of a transposon (48). In both cases, one needs a series of measurements with a luminescent engineered *E. coli* strain to measure luminescence (27). On the other hand, luminescent engineered *E. coli* strains have been used to detect antibiotics by measuring the inhibition of luminescence (48). In both cases, one needs a series of measurements with a luminometer.

Bioassays that rely on simple color observation are the easiest to use by a wide group of users, as evidenced by the commercial use of the Delvotest (Gist-brocades/DSM), which makes use of spore-forming bacteria (*Bacillus steaothermophilus*) to generate a color change after growing in the presence of chromogenic dyes. The sensitivities of the test vary with the antibiotic being assayed. In milk, sensitivities range from 2 to 3,750 ng/g for the fluoroquinolones ciprofloxacin and enrofloxacin. The focal point of the work described here is to generate a color change after growing in the presence of chromogenic dyes. The sensitivities of the test vary with the antibiotic being assayed. In milk, sensitivities range from 2 to 3,750 ng/g for the fluoroquinolones ciprofloxacin and enrofloxacin. The focal point of the work described here is to generate a color change after growing in the presence of chromogenic dyes. The sensitivities of the test vary with the antibiotic being assayed. In milk, sensitivities range from 2 to 3,750 ng/g for the fluoroquinolones cip

<table>
<thead>
<tr>
<th>Strain background</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Relevant phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>J93</td>
<td>Δ(lac)RV</td>
<td>pGEM-T-11</td>
<td>Lac′ Cip′</td>
<td>22</td>
</tr>
<tr>
<td>J93140</td>
<td>lacL</td>
<td>pGEM-T-14</td>
<td>Lac′ Cip′ Tet′</td>
<td>This work</td>
</tr>
<tr>
<td>J93140C</td>
<td>lacI</td>
<td>pGEM-T-14</td>
<td>Lac′ Cip′</td>
<td>This work</td>
</tr>
<tr>
<td>J93140TZ</td>
<td>lacZ</td>
<td>pGEM-T-14</td>
<td>Lac′ Tet′</td>
<td>This work</td>
</tr>
<tr>
<td>BW25113</td>
<td>Δ(arad-araB)567 ΔlacZ4787Δ(araB-araA)368 hsdR514</td>
<td>pGEM-T-11</td>
<td></td>
<td>This work</td>
</tr>
</tbody>
</table>

a For further details, see Materials and Methods.
overnight again (approximately an additional 15 generations). This was repeated several times. Parallel cultures were monitored, and the experiments were repeated several times. Colonies were plated onto LB medium containing 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (see reference 36), and several hundred were typically scored for each culture at each time point. Determination of MIC. MICs were determined according to the method of Andrews (6), except that LB medium was used. An inoculum of 10^6 cells was added to a series of tubes with 1 ml medium with different concentrations of antibiotic. After 18 to 20 h of growth in an incubator at 37°C, the tubes were scored.

**RESULTS**

**Competition experiments.** We constructed isogenic Lac^+ and Lac^- E. coli strain pairs and their ciprofloxacin-resistant (Cip^r) or tetracycline-resistant (Tet^r) derivatives. This enabled us to monitor the percentage of antibiotic-resistant cells in a culture at any point by plating on medium with X-Gal and scoring the white (Lac^-) and blue (Lac^+) colonies. Figure 1 shows an example of this using a Lac^- Cips (ciprofloxacin-sensitive) strain (white) and a Lac^+ Cip^r derivative (blue) mixed together in a ratio of white/blue of between 5:1 and 10:1 and grown in LB medium (Fig. 1A) or LB medium plus Cip at less than one-fifth of the MIC for approximately 25 generations (Fig. 1B). It can be seen that the percentage of blue colonies greatly increases in response to the presence of low levels of Cip.

In order to quantitate these effects more precisely, we grew parallel cultures at different concentrations of Cip and Tet for a different set of isogenic strains (see Materials and Methods). Figure 2 shows the results for Cip, and Fig. 3 shows the results for Tet. It can be seen that in the absence of Cip, the Lac^- Cip^r strain has a growth disadvantage that starts to manifest itself after 15 to 30 generations. Although this is in part due to the Lac^- phenotype, a major part is due to the presence of the mutation conferring resistance to Cip. The resistant mutant was selected spontaneously (see Materials and Methods), and DNA sequencing showed that the respective mutation results in a SER^3 LEU change at position 83 in the gyrA gene, a
mutation that has been found and characterized previously (9, 46). In the strain background used here, this mutation changes the MIC from 16 ng/ml to 350 ng/ml. The effect on growth rates of this allele has been noted previously (9). Because of this effect, low levels of Cip need to overcome the selection in the opposite direction. Although concentrations as low as 1 ng/ml and 2 ng/ml give transient effects (Fig. 2), concentrations of 3 ng/ml (3/16 of the MIC) and higher overcome the reverse selection and allow the resistant cells to completely overtake the population.

The strains used to monitor the change in the percentage of Tet' cells show much smaller changes in competition experiments in the absence of Tet, allowing one to show that concentrations representing 1/20 of the MIC still strongly select for resistant cells in the population (Fig. 3). The variation in parallel cultures does not prevent one from discerning a clear trend. Repeated experiments show a large effect at 62.5 ng/ml Tet but not at 31.25 ng/ml Tet. The MIC for Tet for the strain used is 1,250 ng/ml.

**Construction and use of strains expressing chromogenic proteins.** We engineered strains carrying plasmids encoding some of the newer fluorescent and colored proteins and have used these together with *Escherichia coli* strains carrying gene knockouts that render them more sensitive to one or more antibiotics. The resulting test is a prototype that provides proof of concept for more refined and improved tests. The principle of the test is as follows. Two strains are used together, a wild-type strain and a mutant that is more sensitive than the wild type to a particular antibiotic. Each strain carries a different fluorescent/colored protein. Figure 4 shows the *Escherichia coli* strains we have used for this test. In Fig. 4A, colonies of *E. coli* carrying the plasmid pGEM-T (Promega) encoding either the protein that turns bright yellow in growing cultures or a purple-colored protein (see Materials and Methods). Figure 4B shows the strains in culture. The wild-type strain carries the plasmid encoding the yellow protein, and the mutant strain carries the same plasmid but encoding the purple protein. Figure 5 shows the concept of the test. We engineered the wild-type strain to synthesize the yellow protein and a mutant with a defect in the key efflux system (because of a deletion of the *tolC* gene) to synthesize the purple protein. The latter strain is more sensitive to various antibiotics than the wild type. The two strains are mixed together with a ratio of mutant (purple)/wild type (yellow) of more than 20:1. This is to allow the mixture, when grown in broth without an antibiotic, to be purple, since the small amount of yellow wild type does not quench the purple color to a significant degree. However, when grown in broth containing an antibiotic to which the *tolC* mutant is more sensitive than the wild type and at a concentration that is subinhibitory to the wild type, the mutant is inhibited. Even if the inhibition is not complete, the wild type will outgrow the mutant during the time course of the test. Figure 6 shows the results of seeding a set of broth cultures with the

**FIG. 4.** Chromogenic proteins. Colonies and cultures of BW25113 carrying pGEM-T-11 or pGEM-T-14 are yellow or purple, respectively.

**FIG. 5.** Concept of color test for detection of antibiotics and genotoxic agents. WT, wild type.
same mixture of the wild type and mutant, with each tube containing concentrations of ciprofloxacin (Cip) ranging from 0 to 5 ng/ml. The tube on the far left contains broth with no Cip, the second tube 1 ng/ml Cip, and the third, fourth, fifth and sixth tubes 2, 3, 4, and 5 ng/ml Cip, respectively. It can be seen that at 0 and 1 ng/ml Cip, the tubes remain purple, since neither the wild type or the mutant are preferentially inhibited. However, already at 2 ng/ml Cip, one can see that the cultures turn bright yellow, because even at this concentration, only the tolC mutant is inhibited since it is 1 order of magnitude more sensitive than the wild type. The MIC for the wild type used here is between 16 and 20 ng/ml Cip.

The effects are not specific for Cip, since the mutant used is more sensitive to a battery of different antibiotics (32, 46). We applied the same test to tetracycline (Tet), fusidic acid (Fus), and erythromycin (Ery). The results are shown in Table 2. This test detects 50 ng/ml Tet, 500 ng/ml Fus, and between 625 and 1,250 ng/ml Ery.

**Engineering hypersensitive strains with multiple mutants.** We have shown in recent work (46) that combining gene knockouts that affect sensitivity to an antibiotic via different pathways can result in a strain that is more sensitive than strains resulting from either single knockout. Therefore, to increase the sensitivity of the chromogenic test, we used a double knockout with deletions of both the tolC gene and the recC gene, since our experiments have shown that while tolC or recC alone reduce the MIC for Cip from 16 ng/ml to 2 to 4 ng/ml, the double mutant displayed a MIC of 0.3 ng/ml (46). Figure 7 shows the results of using this strain with the yellow chromogenic marker in place of the single tolC mutant used in Fig. 4 and 6. Here, the concentrations of Cip, reading from the left, are 0, 0.05, 0.1, 0.2, 0.5, and 1 ng/ml. Clearly, one can detect a significant reaction at 0.2 ng (200 pg/ml).

We can use different combinations of sensitive mutations to target not only different antibiotics but also genotoxic agents. We found that strains carrying the double mutant uvrA recO are hypersensitive to the antibiotic and DNA-damaging agent nitrofurantoin (NIT) (10) and that strains with the double mutant uvrA recG are hypersensitive to the antitumor DNA cross-linking agent cisplatin (CPT) (10). We therefore transformed the plasmid (pGEM14) carrying the gene conferring the purple color phenotype into these double mutant strains and demonstrated that they can be incorporated into the same test. Table 3 shows the results. One can detect levels of NIT at 100 ng/ml, 1/20 of the MIC, and of CPT at 25 μg/ml, 1/4 of the MIC.

**Plate test.** We have also developed a plate version of this test that can be used to screen for compounds that specifically inhibit one strain or mutant with respect to a second strain. Using the same principle as shown in Fig. 5, a mixture of two colored strains (here, a 50:1 ratio of sensitive purple to less-sensitive yellow) with different susceptibilities to a given antibiotic will generate a lawn on an LB plate with the color of the majority, in this case, purple. An antibiotic applied to the plate as a drop or disc when the cells are first plated will generate a clearing of the resulting lawn at the point of application, as the antibiotic diffuses, a concentration gradient occurs, such that at low concentrations at the outer edges of the plate, the cells grow normally and are purple. However, at some point, the concent-

### Table 2. Detection limits for Tet, Fus, and Ery

<table>
<thead>
<tr>
<th>Drug</th>
<th>Detection level(s) (ng/ml)</th>
<th>WT MIC (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tet</td>
<td>50</td>
<td>$2.5 \times 10^3$</td>
</tr>
<tr>
<td>Fus</td>
<td>500</td>
<td>$3 \times 10^5$</td>
</tr>
<tr>
<td>Ery</td>
<td>625–1,250</td>
<td>$1.5 \times 10^5$</td>
</tr>
</tbody>
</table>

*The mutant genotype for Tet, Fus, and Ery is ΔtolC. WT, wild type.

### Table 3. Detection limits for CPT and NIT

<table>
<thead>
<tr>
<th>Drug</th>
<th>Detection level (μg/ml)</th>
<th>WT MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>NIT</td>
<td>0.1</td>
<td>2</td>
</tr>
</tbody>
</table>

*The mutant genotype for both CPT and NIT is ΔrecG::kan uvrA::tet. WT, wild type.
tration of the antibiotic is high enough for the mutant strain to be sensitive but not high enough to inhibit the wild type, so in this range, the cells that grow generate yellow colonies. The net result is a ring of yellow surrounded by a lawn of purple. Figure 8 shows this for Cip applied to the center of a plate spread with a mixture of wild-type cells (purple) and tolC recC cells (yellow). In the example shown here, a drop of a solution with 1,000 ng/ml Cip was used, although the detection limit for this test is at or below 20 ng/ml Cip. Additionally, the photograph of this plate (using a Canon 8.1-megapixel camera) was subjected to density analysis using ImageJ software, which illustrated an ~87% difference in grayscale intensity between the inner yellow ring and the rest of the plate.

**DISCUSSION**

The proliferation of multidrug-resistant pathogens is a major problem in public health. More people in the United States die each year from MRSA (methicillin-resistant Staphylococcus aureus) than from AIDS, according to data from 2006 (11, 12). The exposure to low levels of antibiotics is one of several causes of the spread of resistant microorganisms (30). Studies have shown that trace levels of antibiotics can activate transcription of numerous operons in bacteria (24, 51, 52). Examination of the human intestinal microbiome reveals a reservoir of microorganisms that are resistant to many of the antibiotics in use (44). Here we use competition tests between wild-type and resistant strains of E. coli to show that low levels of Cip (<1/5 of the MIC) or Tet (1/20 of the MIC) select for the outgrowth of resistant cells, underscoring the need to reduce the exposure to even very low levels of antibiotics. Some alleles, such as those conferring resistance to Cip, result in a loss of fitness relative to that of the wild type in the absence of antibiotic (0 ng/ml Cip) (Fig. 2) (see also reference 4), a phenomenon that has been well documented (e.g., see references 4, 5, 20, 26, 38, and 39). This underscores the value of reducing the exposure to antibiotics. Studies have shown that lowering the amount of antibiotics given to farm animals results in a measurable reduction in antibiotic-resistant microorganisms (1).

We also present a simple test that does not require expensive equipment to monitor the levels of different antibiotics. This test is already significantly more sensitive for Cip than many of the simpler tests, such as the Delvotest, the Copan milk test, the Brilliant black reduction test, the Eclipse 100 test, and the blue-yellow test (31, 41). Future work will be aimed at increasing the sensitivity of the test even further by different strategies, such as examining targeted triple mutants that lack three different functions involved in intrinsic resistance (e.g., repair, efflux, and protein synthesis functions) that we have identified in a recent study (32), using a synergistic codrug, such as an aminoglycoside in the case of Cip (see reference 50), or measuring the fluorescence of the respective marked strains. (Chait and coworkers have used fluorescence in a differential plate assay for compounds that select against antibiotic resistance [13]). It is possible to engineer combinations of mutations that yield specific sensitivities or resistance to different antibiotics, and we have done this for the DNA-damaging agents nitrofurantoin (NIT) and cisplatin (CPT) (Table 3). Methods for
markerless deletion construction (8, 21, 53) could be used to convert these strains to derivatives lacking the antibiotic resistance markers that are associated with each deletion mutation. One might construct nonplasmid versions of the tester strains by moving the gene encoding the chromogenic proteins from the plasmid to the chromosome by techniques that have been developed for this precise purpose (37, 40).

ACKNOWLEDGMENT

This work was supported by a grant from the National Institutes of Health (ES0110875).

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