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Evolution of altruists and cheaters in near-isogenic populations of *Escherichia coli*

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1. ABSTRACT

Emergence of antibiotic-resistant bacteria threatens the continued efficacy of many critical drugs used to treat serious infections. What if such resistant organisms could also act as altruists and "share" their resistance with sensitive cohorts without any actual genetic exchange? We competed resistant strains that differ solely in their ability to secrete a plasmid-encoded beta-lactamase. Sensitive strains were otherwise isogenic with their resistant counterparts and were either plasmid-free or contained a "Dummy" plasmid of roughly the same size as that of the resistance plasmids. Absent antibiotic selection, plasmid-free sensitive strains outperformed the plasmid-bearing strains. In the presence of ampicillin, the outcome depended on whether the resistant strain secreted its beta-lactamase (Altruist) or retained it (Selfish). In the latter case, only resistant cells survived. When beta-lactamase was secreted, some sensitive cohorts were also provided protection, with the largest fitness increase provided to plasmid-free cells. However, some Altruist strains appeared to be at a disadvantage, as a great deal of their enzyme broke off cells. Thus, additional variables must be considered when designing microbial competition experiments.

2. INTRODUCTION

One can examine cooperation between individuals in a variety of contexts. In groups, some individuals may possess a characteristic that provides a benefit to the group as a whole, or at least to some members of the group, thus improving the fitness of the group as a whole. If such individuals produce a benefit to others without receiving a reciprocal benefit, then they are often labeled "Altruists" in the evolutionary literature. In contrast, "Selfish" individuals possess a useful characteristic, but only they benefit. In the presence of Altruists, a third type of individual called "Cheaters" may emerge that benefit from the presence of Altruists in the group, without making a contribution to the group as a whole (1). We have explored this process with near-isogenic bacteria, where we could better control the variables involved in such behavior (1-3).

An important characteristic of bacteria is their potential role(s) in disease production. In this context, the evolution of antibiotic resistance is particularly important, both from a theoretical and practical standpoint. Such evolution often takes advantage of genes and pathways that serve as reservoirs for development of resistance to

antibiotics, new and old, and the mechanisms of resistance often may be horizontally transferred along with the respective genes responsible (4,5; for reviews, see 6,7). However, some environments lend themselves to patterns of cooperation among the microbes. Such cooperation has been shown, at times, to take the form of some members of the population providing protection to others (8). In biofilms, this has included protection against toxic chemicals and antimicrobials (9-11). Protection against antibiotics has also been seen in free-floating communities (8, 12-14). For instance, it has long been observed in gram-positive bacteria that an "inoculum effect" exists with respect to resistance to beta-lactam antibiotics, such as penicillins (e.g., ampicillin; 15,16). Due to the nature of gram-positive cell structure, the proteins responsible for such decreased resistance, beta-lactamases, are typically found as high-affinity enzymes secreted into the environment. Thus, the more of these bacteria that are present in the growth medium, the greater the available pool of enzyme to destroy the substrate antibiotics and allow the bacteria to survive. In principle, there is no reason why this benefit might not also be obtained by otherwise antibiotic-susceptible organisms in the same medium. Such bacteria could be considered "Cheaters" since they benefit, ostensibly without contributing to the resistance effort. But in this case, the ability of Cheaters to emerge implies a frequency dependence since before Cheaters could survive, a minimum number of Altruists would be necessary and, beyond this threshold, the number of Cheaters would likely be directly related to the number of Altruists in the group.

We have explored this dynamic previously, both experimentally and by developing theoretical models (1-3). In the studies described here several hypotheses were tested, as were several of the assumptions made in designing the competitions between ampicillin-resistant strains and their sensitive cohorts. First, it was predicted that sensitive strains would not survive in the presence of ampicillin when grown together with "Selfish" strains; i.e., "Selfish" strains could not help the sensitive individuals survive in the presence of antibiotic. Next, plasmid-free cells and "Dummy" cells (i.e., bearing a plasmid of the same size and approximate cost as found in the Altruist and Selfish strains, but without conferring ampicillin resistance) would survive in the presence of ampicillin if "Altruists" were present at a minimum concentration. Finally, the "Altruists" would provide a greater benefit to plasmid-free strains than to "Dummy" strains. In addition, we wanted to fully consider the variables that affect the outcomes in such competitions. For example, our previous work allowed comparison of group benefit provided in shaking cultures compared with stationary or biofilm-like settings (1). However, one could also consider how often, if ever, cultures were exchanged for waste products and with fresh nutrients. This comparison was made since we discovered that in the apparatus used previously for such competitions (1) no detectable movement occurred across the dialysis tubing used to separate the cell culture side from the fresh nutrient side. Further, we tested assumptions regarding equivalent growth rates among the plasmid-bearing strains, the location of beta-lactamase in the antibiotic resistant cells and the levels of ampicillin maintained during competitions.

3. MATERIALS AND METHODS

3.1. Host Bacterial Strains and Plasmids

For these studies we used strains that were otherwise isogenic, but that could be distinguished visually on agar plates due to color differences (e.g., in the presence of chromogenic substrates such as X-gal). KL99 (HfrH, LAM^- *relA1spoT1,thi1*; *E. coli* Stock Center, CGSC, Yale University) was a wild type progenitor strain and was $LacZ^+$, whereas strain 5240 (HfrH, LAM^- *lacZ43(fs)relA1spoT1,thi1*; *E. coli* Stock Center, CGSC, Yale University) was otherwise isogenic with KL99, but was $LacZ^-$, due to a frameshift mutation at position 43. It was important that these strains had similar growth rates, and this was an aspect of their characteristics that was assessed in the set of experiments prior to competition studies.

The plasmids used have been described previously (1). All plasmids are derived from pCR2.1 TOPO (Invitrogen, Carlsbad, CA) modified so that the only source of ampicillin resistance that can be provided by any plasmid is that originating from a derivative of the TEM-1 beta-lactamase gene cloned into the multiple cloning site and placed under the control of the *lac* promoter of the vector: Altruist (pSAR1; 1) and Selfish (pSLAR1; 1). The control plasmid, Dummy (pAmpsens1; 1), is the same size as the other two and includes the *aphA-2* gene encoding resistance to aminoglycosides such as kanamycin, instead of the beta-lactamase gene. Thus, this plasmid presumably yields about the same cellular cost, but lacks the ability to confer resistance to beta-lactam antibiotics like ampicillin.

3.2. Measurement of beta-lactamase activity and ampicillin concentrations

Beta-lactamase activity was measured using a spectrophometric assay (17) with nitrocefin (Glaxo Research, Oxoid,UK) as the chromogenic cephalosporin substrate. The color change from yellow to red when nitrocefin was cleaved by beta-lactamase was monitored over a 30 min period at 495 nm. Activity was measured from whole cells grown in liquid culture, supernatants of such cultures after centrifugation to collect cells, and sonic extracts of pelleted cells. In the latter two cases, activities were normalized to amount of protein, as measured by Bradford assay (18; Bio-Rad, Hercules, CA). Ampicillin concentrations remaining in culture media were measured by the Folin-Ciocalteu method (19), using ampicillin standards in Davis Minimal Medium (DMM; 20) for the spectrophotometric assay.

3.3. Conditions for growth and competitions

A dual-flask system (Bellco BIOTECHNOLOGY,Vineland, NJ) was used in these experiments (Figure 1). The filter used between the two flasks was a Miracloth - 0.2 μm Supor-200 membrane (PALL) sandwich; in earlier experiments (1) dialysis tubing was used between the flasks. The junction between the two flasks was sealed with automotive/plumber's Teflon tape.

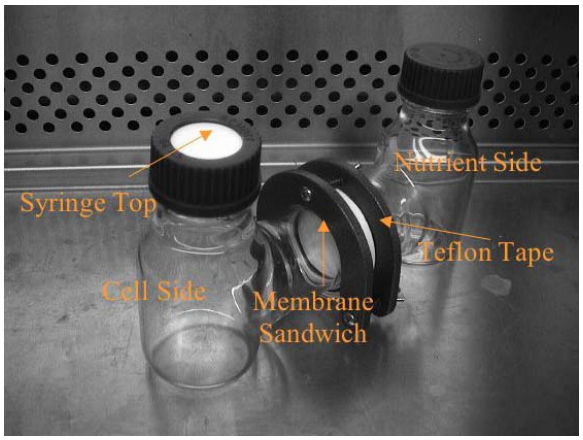

Figure 1. Dual-flask system used for competition experiments. Competitions were carried out in flask set-ups as above, to allow regular replenishment of nutrients and ampicillin. Cells were inoculated into 100 ml DMM medium (20) on the "Cell Side" with or without ampicillin, as required for a particular competition. The same medium was added to the "Nutrient Side" and cultures were shaken at 37 °C for 12 h. Aliquots were taken via a sterile 18g needle and syringe through the "Syringe Top" enclosure, and used for measurements of cell numbers, characterization of plasmid content, antibiotic resistance/susceptibilities, etc. The growth medium on the "Nutrient Side" was sterilely removed and replaced with fresh medium of the same type prior to additional incubation/shaking at 37 °C.

This set up allowed for dilution of waste products, maintenance of proper nutrient and drug concentrations, and less opportunity for contamination. To test whether waste products and nutrients were able to cross the Miracloth/0.2 micron filter "sandwich", while whole cells were prevented from crossing from one side to the other, the flasks were inoculated with cells in DMM containing ampicillin on the "Cell" side and with the same medium absent cells on the "Nutrient" side. Both sides were assayed for beta-lactamase activity and ampicillin concentration. Also, both sides were plated onto LB agar containing X-gal and IPTG. In competitions, both strains were diluted to the desired ratio (either 50:50 or 75 resistant:25 sensitive) and inoculated on the "Cell" side into DMM, supplemented with dextrose (0.006%), MgSO4 (1 mM), thiamine (0.0005%), Casamino acids (0.004%), and IPTG (0.16 mM), with or without ampicillin. All competitions were run for at least 120 h, were done in duplicate, and were repeated with each genetic background and plasmid combination. Flasks were re-fed on the "Nutrient" side (i.e., with no bacteria) every 12 h with fresh media. Samples were taken every 12 h and analyzed by measuring the A_{600} and by dilution plating, by spotting 40 microliters of 10^5 and 10^6 dilutions onto XI agar (containing X-gal (80 micrograms/ml) + IPTG (0.16 mM)), AXI agar (XI + 100 micrograms ampicillin per ml), and, when the Dummy strain was used, KXI agar (XI + 50 micrograms kanamycin per ml). Three spots were counted for each dilution, comparing numbers of white vs. blue colonies.

4. RESULTS

4.1. Testing membrane selective permeability of competition flasks: whole cells do not cross, while beta-lactamase and ampicillin cross freely

Whole cells did not cross the membrane, as no cells were found when plating aliquots from the "Nutrient" side of flasks. Levels of beta-lactamase were measured for ampicillin-resistant strains (Altruists, Selfish) grown in competition flasks in the presence of 100 micrograms ampicillin per ml, using the spectrophotometric assay over 30 min, with nitrocefin. A separate spectrophotometric assay measured ampicillin levels on both sides of the competition flasks. In contrast to what was seen with whole cells, concentrations of both beta-lactamase and ampicillin became equal on both sides of the membrane and remained so (data not shown).

4.2. Growth rates of starting strains are approximately the same, but all plasmid-bearing strains are not "created equal"

Growth curves were used to compare all strains. The initial background, plasmid-free strains, KL99 and 5240, were grown either separately or together (50:50) in DMM medium without ampicillin. As seen in Figure 2, both plasmid-free strains grew at approximately equal rates. However, when the strains contained plasmids some differences were observed in growth rates. For some independently isolated transformants of 5240 containing the Altruist plasmid, the strains grew significantly more slowly in 100 μg ampicillin per ml than the corresponding strain containing either the Selfish or Dummy plasmids and, more slowly than the KL99 strain carrying either plasmid (Figure 3A). While either background strain bearing the Selfish plasmid and KL99 bearing the Altruist plasmid had reached late log phase by 12 h post inoculation, one transformed 5240 strain (designated 5240 Alt3), for example, typically required 14 h to reach mid-log phase. If this isolate was given a 2 h "head-start" prior to inoculation of the other strains in growth curve studies, the strain then reach mid-log phase at approximately the same period as the others (Figure 3A, inset). On the other hand, other transformants of 5240 carrying this plasmid grew equally as well as all the other strains under these conditions. No obvious difference was detected between the different 5240 transformants bearing the Altruist plasmid in terms of plasmid size. Interestingly, after repeated re-streaking of the 5240 Alt3 strain on agar containing ampicillin, the strain began to show more or less the same growth rate as the other strains in the study and no longer required the 2 h head start (Figure 3B). Whole genome *E. coli* microarrays are currently being used in our lab to compare global gene expression between the original 5240 Alt strain (from frozen stock) and the adapted, more rapidly-growing version.

4.3. Competitions to determine whether background strains bearing the different plasmids grow equally well in the presence of antibiotic

To determine whether background strains to be used for later competitions grew equally well when bearing the same plasmids, strains were initially inoculated at equal

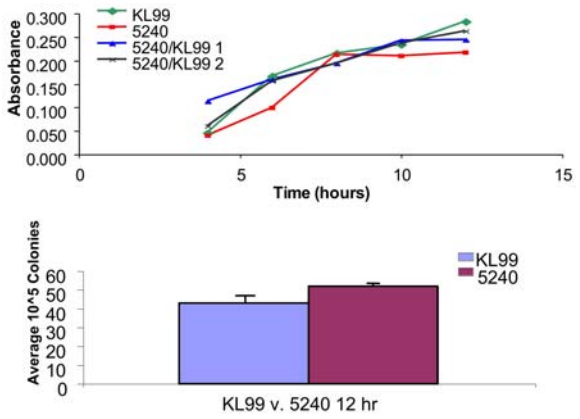

Figure 2. Growth rates of background strains are approximately equal. Strains 5240 and KL99 were grown either separately or together (initial proportions 50:50), in competition flasks in DMM supplemented as described in Materials and Methods. Growth was followed spectrophotometrically at 600 nm and by plating samples to count colonies. The data shown here reflect competition results from cultures grown together and sampled at 12 h. Measures reflect the average numbers of colonies (x 10^5) for each strain. In lower graph, Blue, KL99; White, 5240.

proportions in competition flasks and were sampled at 12 h. As seen in Figure 4A, the expectation of equal growth rates was in fact borne out for both the Altruist and Dummy plasmids, while the KL99 strain bearing the Selfish plasmid had a significant growth advantage over the corresponding 5240 strain with the same plasmid. Another assumption of the experimental design in these experiments was that all plasmids should confer approximately the same cost on host cells. To investigate this assumption, strains bearing the Altruist plasmid were competed against their counterparts bearing the Selfish plasmid. The strains had approximately the same growth rates, although there appeared to be a slight advantage for either plasmid in the KL99 background (Figure 4B)

4.4. Testing beta-lactamase location in liquid cultures of resistant strains

Assays were carried out on pelleted whole cells, supernatants from the centrifuged cultures, and sonicated cell pellets. In the latter two cases, amounts of beta-lactamase activity were normalized to amount of protein, whereas activity for whole cells was normalized to wet weight of cells pelleted. As seen in Figure 5, roughly six times as much beta-lactamase was found in the supernatant from cells bearing the Altruist plasmid compared with those bearing the Selfish plasmid. Moreover, the activity associated with whole cells for the Altruist-bearing cells was approximately three times that for the corresponding cells with the Selfish plasmid (not shown). Overall these results are consistent with the expected cellular localization of the beta-lactamase for the respective cell types, but additionally they show that a considerable amount of beta-lactamase is released or broken off into the surrounding supernatant during growth of the Altruist cells.

4.5. Competitions for 120 h

Once strains in this study had been characterized in terms of their initial growth rates, beta-lactamase production, and plasmid content, competitions were conducted with a minimum of two biological replicates, in the form of independent competitions in separate flask set-ups. Initial competitions were conducted with starting ratios of competitors at equal proportions (50:50). Additional competitions were conducted with starting ratios of 75% ampicillin-resistant competitor vs. 25% ampicillin-sensitive strain. Figure 6 shows the results of representative competitions in the absence of ampicillin. For all these competitions, plasmid-free strains out-competed all plasmid-bearing strains in direct competitions. Depending on the background strain and the particular plasmid, strains bearing the Selfish or Altruist plasmids dropped to between 6 and 31% (Figure 6A). Similarly, strains bearing the Dummy plasmid were also at a disadvantage in medium without antibiotic selection for the plasmid (Figure 6B).

In the presence of ampicillin, only cells bearing the Altruist or Selfish plasmids would be expected to survive when only considering which cells produce beta-lactamase. The Dummy strains bear approximately the same cost as the Selfish and Altruist strains, but lack the ability to produce beta-lactamase. When either Altruist or Selfish strains were competed against strains bearing the Dummy plasmid (Figure 7A), regardless of whether the initial ratio of resistant to susceptible cells was 50:50 or 75:25, no Dummy cell survivors were observed. Likewise, when Selfish strains were competed against plasmid-free strains in the absence of ampicillin, only Selfish cells survived (Figure 7B).

A crucial prediction of our models (2, 3), previous experimental work (1), and experimental design for the current experiments is that the Altruist cells should cause degradation of ampicillin in their immediate vicinity, protecting not only themselves from the action of the drug, but also potentially aiding the survival of normally ampicillin-susceptible cells nearby. In fact, survivors of the plasmid-free strains appeared when Altruist strains were competed against them in the presence of 100 micrograms ampicillin per ml. These plasmid-free isolates can be termed Cheaters, since they have taken advantage of the beta-lactamase provided by their neighboring Altruist cells. Moreover, the number (and overall proportion) of Cheaters increased when the initial proportion of Altruist cells was increased from 50% to 75% (Figure 8). Such survivors were truly Cheaters: when plated from liquid culture onto agar containing the same concentration of ampicillin, they remained ampicillin susceptible.

4.6. Other kinds of cheaters

When the KL99 strain bearing the Selfish plasmid was grown in competition with plasmid-free 5240, in the absence of ampicillin, cells were plated on XI and AXI plates and the numbers of blue and white colonies were compared. Numbers of the blue (KL99 Selfish) strain were significantly reduced, with the overall number of colonies on the AXI plates being roughly 2-fold less compared to those on XI (i.e., without ampicillin)(Figure

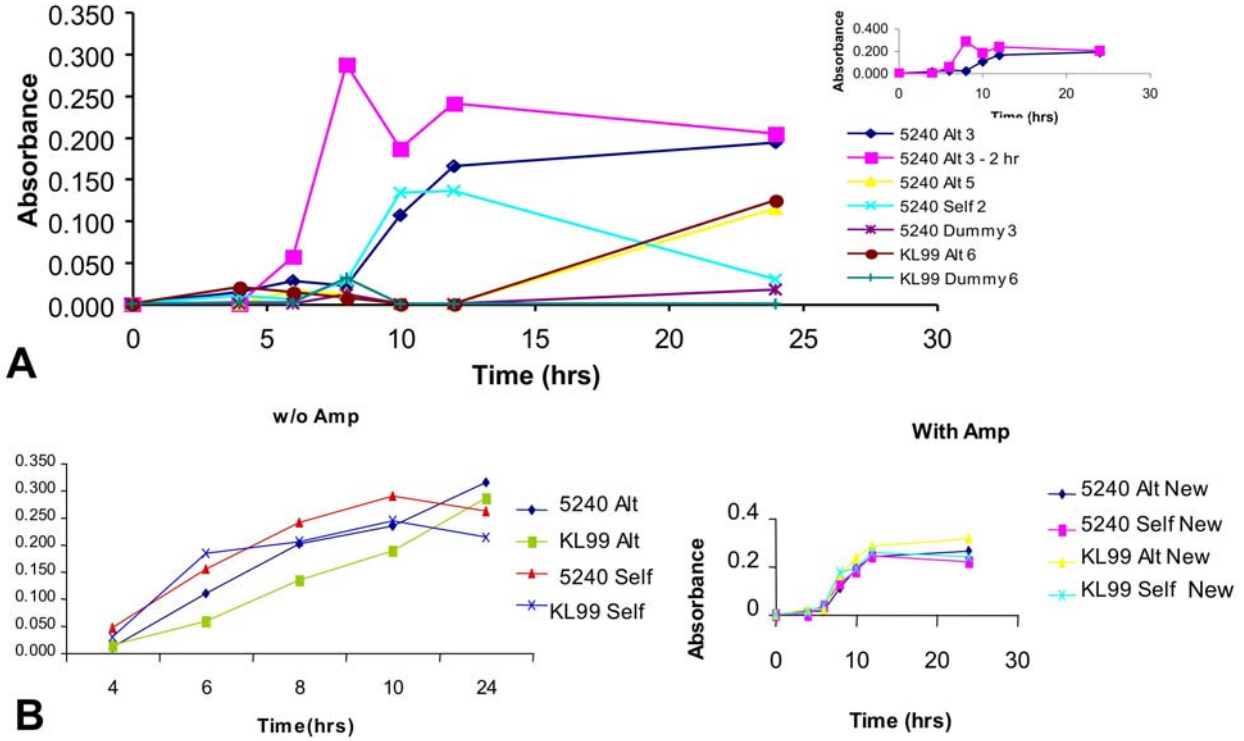

Figure 3. Growth rates for strains with plasmids. Growth curves measuring absorbance at 600 nm were carried out comparing different 5240 and KL99 transformants bearing either the Altruist (Alt) or Selfish (Self) plasmids, grown in the presence of 100 micrograms ampicillin per ml. Panel A, Initial isolate comparisons in presence of ampicillin; inset, growth curve showing that 5240 Alt3 "catches up" with other strains if given a 2 h head-start. Panel B, Growth curves for strains used in further competitions.. Upper graph, grown without ampicillin; lower graph, grown with ampicillin.

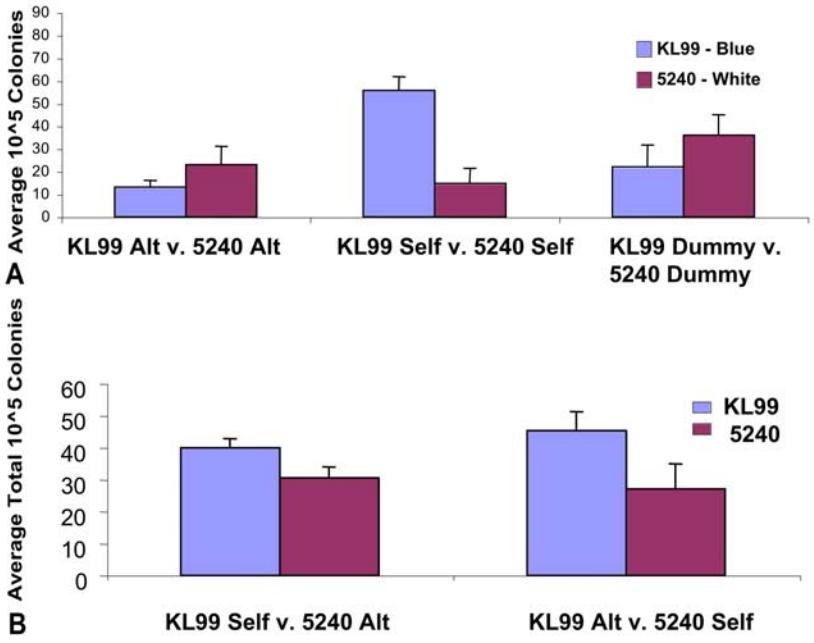

Figure 4. Comparison of strains bearing different plasmids. Relative growth of strains in competition when A) different background strains bore the same plasmid or, B) when one background strain bore the Altruist plasmid and the other bore the Selfish plasmid. Strains were grown together in competition flasks with ampicillin and sampled at 12 h for plating to count relative numbers of colonies.

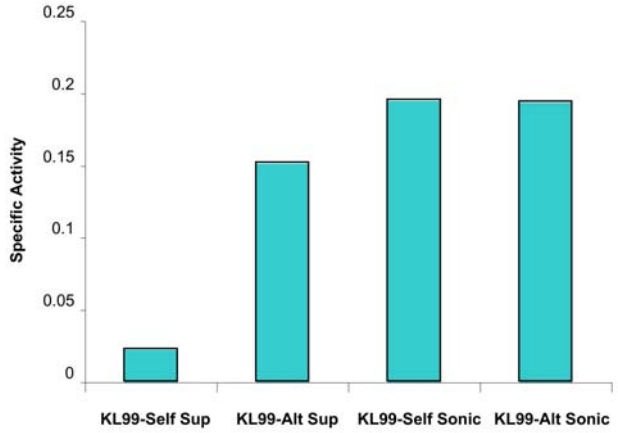

Figure 5. Beta-lactamase activity localization. Beta-lactamase activity was determined using the spectrophotometric assay (17) with nitrocefin as the chromogenic substrate. Activity was measured from cells grown for 12 h in competition flasks in DMM supplemented with 100 micrograms ampicillin per ml and other nutrients as described in Materials and Methods. Supernatant from pelleted cells, whole cells, and sonic extracts of whole cells were each assayed. Specific activity was calculated for the supernatants and sonic extracts by normalizing changes in amount of nitrocefin to amount of protein present. For whole cells (not shown), levels were expressed as change in absorbance at 495 nm. Data are presented for KL99 bearing the Altruist or Selfish plasmids; similar results (not shown) were obtained for the corresponding strains in the 5240 background.

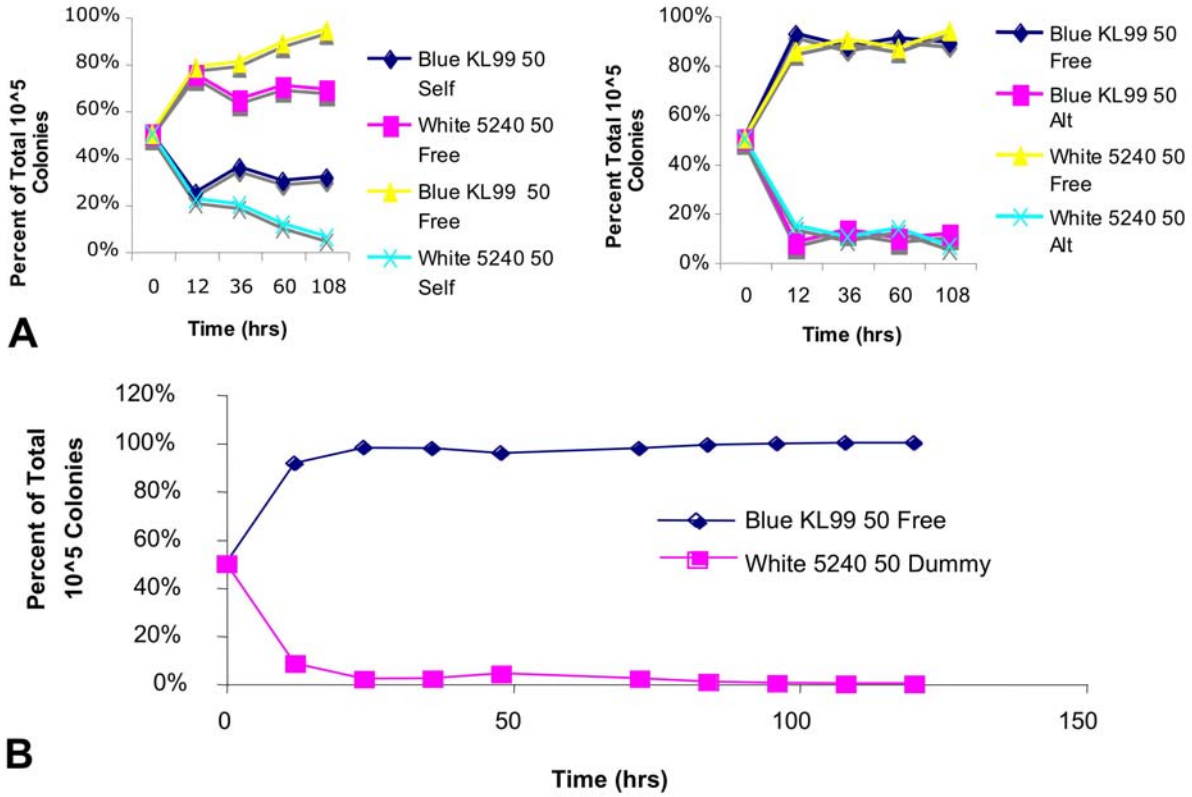

Figure 6. Competitions between plasmid-bearing and plasmid-free strains in the absence of ampicillin. Strains bearing either the Altruist or Selfish plasmids were competed against their plasmid-free counterparts over the course of 108 h without ampicillin. The proportions of blue and white colonies were determined for each competition and these are presented in the graphs. All competitions were initiated at 50:50 proportion for each strain. Panel A, KL99 bearing Selfish plasmid vs. plasmid-free 5240; 5240 bearing Selfish plasmid vs. plasmid-free KL99; KL99 bearing Altruist plasmid vs. plasmid-free 5240; 5240 bearing Altruist plasmid vs. plasmid-free KL99. Panel B, plasmid-free KL99 vs. 5240 bearing the Dummy plasmid.

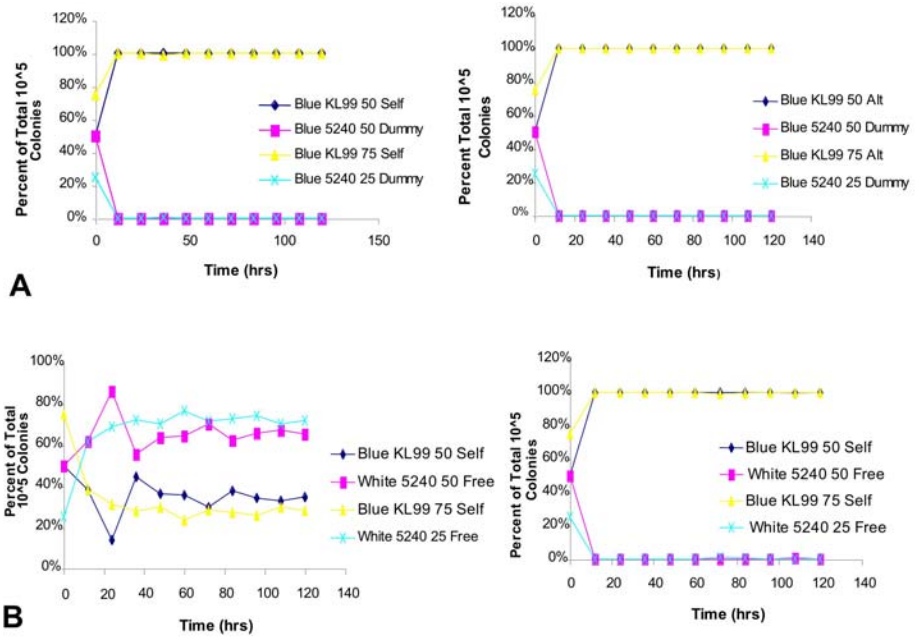

Figure 7. Competitions where no Cheaters were observed. Strains bearing either the Altruist or Selfish plasmids were competed against their counterparts bearing the Dummy plasmid over the course of 108 h in the presence of 100 micrograms ampicillin per ml DMM. The proportions of blue and white colonies were determined for each competition and these are presented in the graphs. All competitions were initiated at 50:50 proportion and separately at 75% resistant:25% susceptible for each strain. A, KL99 bearing Selfish plasmid vs. 5240 bearing the Dummy plasmid, upper graph; KL99 bearing Altruist plasmid vs. 5240 bearing the Dummy plasmid, lower graph. B, KL99 bearing Selfish plasmid vs. plasmid-free 5240 in the absence (upper graph) or presence (lower graph) of 100 micrograms ampicillin per ml DMM.

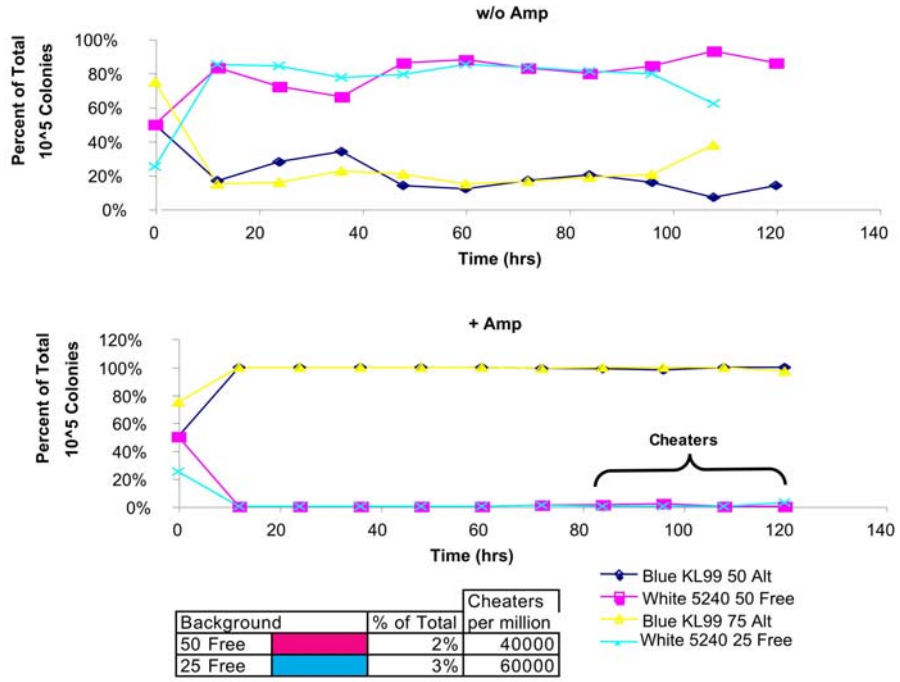

Figure 8. Cheaters are only observed when strains bearing the Altruist plasmid compete against plasmid-free strains. Strains bearing the Altruist plasmid were competed against their plasmid-free counterparts over the course of 120 h in the presence of 100 micrograms ampicillin per ml DMM. The proportions of blue and white colonies were determined for each competition and these are presented in the graphs. All competitions were initiated at 50:50 proportion and separately at 75% resistant:25% susceptible for each strain. KL99 bearing Altruist plasmid vs. plasmid-free 5240 in the absence (upper graph) or presence (lower graph) of 100 micrograms ampicillin per ml DMM.

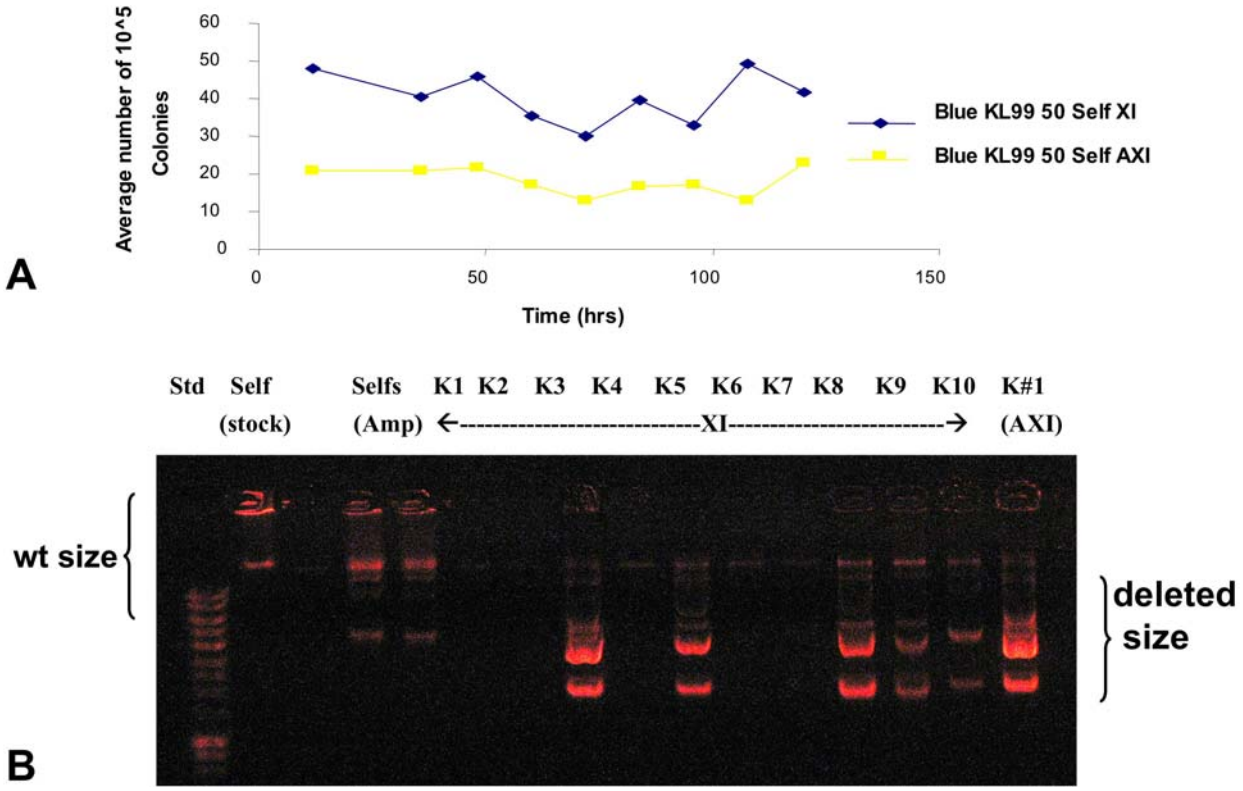

Figure 9. Other types of cheaters: Selfs lose plasmid or shorten plasmids; Alts can become cheaters too. From a competition between KL99 bearing the Selfish plasmid and 5240 (plasmid-free) cells grown in the absence of drug, colonies were produced (Panel A) on XI (upper, blue curve) and AXI plates (lower, yellow curve). For the agarose gel in Panel B, plasmid DNA preparations K1-K10 were from blue colonies that grew originally on XI, while K#1 was from a blue colony taken off AXI. Self (stock), plasmid obtained from the progenitor KL99Self strain; Selfs (Amp), plasmid from blue colonies after KL99Self competition in liquid DMM containing 100 micrograms ampicillin per ml. Std, linear size standard, Hyper Ladder I (BIOLINE).

9A). In order to evaluate the basis for this difference, blue colonies from the XI plates were screened for plasmid content and size. As seen in Figure 9B, in the absence of drug, some KL99 cells lost the Selfish plasmid or generated deleted forms. These cells would thus be expected to have reduced cost relative to their progenitor strains and could thereby compete more effectively against the plasmid-free 5240 cells in the absence of drug.

One could similarly ask whether another type of Cheater would emerge from Altruist cells that have, similarly, lost their plasmids during competitions in the presence of ampicillin. In fact, a presumed Altruist colony chosen randomly from a spot containing plasmid-free Cheaters, was also found to lack plasmid and did not grow on AXI (not shown).

5. DISCUSSION

In this report we have extended previous studies that utilized a bacterial model for altruistic behavior, leading to survival of otherwise antibiotic-susceptible cohorts (1). The current study utilized a competition flask system with much greater exchange of fresh nutrients and antibiotic than we had used previously. As discussed later, this may explain, in part, some slightly different outcomes obtained here. In addition, in the current study, location and levels of beta-lactamase in the strains used was thoroughly tracked.

We expected plasmid-bearing strains to be less fit compared to plasmid-free strains, if no antibiotic were present. Interestingly, we noticed that in some cases, strains bearing the Altruist plasmid had a significant growth disadvantage, even compared to strains bearing other plasmids. In part this could be due to perturbations of the outer membrane due to the Lpp-OmpA-Bla tripartite fusion (21). This may also be related to the finding here that significant amounts of beta-lactamase break off the surface of Altruist cells (see Figure 5). Lattemann et al. (22) found that the OmpT protease similarly released a certain proportion of beta-lactamase from the cell surface and that such release was reduced in *ompT* mutant cells.

Here we have confirmed previous work (1, 23) showing that bearing a plasmid carries a cost that makes such strains less competitive compared to their plasmid-free cohorts. Cells with the Dummy plasmid were at a disadvantage compared to plasmid-free strains. Most importantly, cells with the Dummy plasmid did not yield

Cheaters when competed with Altruists in this system. However, some plasmid-free background strains were Cheaters when competed with Altruists. In terms of overall numbers of Cheaters, less were found in the present study than in our previous work (1). In part, this is likely due to differences in the competition flask set-up. The set-up used in previous experiments (1) did not allow free exchange of fresh ampicillin or beta-lactamase between the "Cell" side and the fresh "Nutrient" side. Thus, it is likely that beta-lactamase built up on the "Cell" side during the course of competitions and that ampicillin concentrations diminished. In contrast, here we showed that ampicillin and beta-lactamase exchanged freely between both sides of the new apparatus. This, combined with 12 h exchanges of fresh medium (and ampicillin) tended both to dilute beta-lactamase and maintain ampicillin concentrations throughout the competitions. This regime likely more closely mimics that seen during outpatient antibiotic treatment for bacterial infections. Furthermore, we found here that the burden of maintaining a plasmid could be overcome. When grown without drug, some cells lost their Selfish plasmids, while others contained deleted versions of the plasmid, presumably with lower cost, especially for competitions in the absence of drug. Importantly, there was more than one path to yield Cheaters during antibiotic selection. Plasmid-free cells were found to survive in the presence of Altruists. Moreover, some Altruist cells lost their plasmids and survived in the presence of plasmid-bearing Altruists. The implications of these findings on the evolution of antibiotic resistance, survival in otherwise lethal concentrations of drug, and the maintenance of diversity in near-isogenic populations of bacteria are plentiful.

6. ACKNOWLEDGMENTS

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Abbreviations: DMM, Davis Minimal Medium; IPTG, ; X-gal, ; XI, LB agar containing X-gal + IPTG; AXI, LB agar containing 100 micrograms ampicillin per ml and X-gal + IPTG; KXI agar, XI + 50 micrograms kanamycin per ml.

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