Protection of *Salmonella* by ampicillin-resistant *Escherichia coli* in the presence of otherwise lethal drug concentrations

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Microbial systems have become the preferred testing grounds for experimental work on the evolution of traits that benefit other group members. This work, based on conceptual and theoretical models of frequency-dependent selection within populations, has proven fruitful in terms of understanding the dynamics of group beneficial or ‘public goods’ traits *within species*. Here, we expand the scope of microbial work on the evolution of group-beneficial traits to the case of *multi-species communities*, particularly those that affect human health. We examined whether β -lactamase-producing *Escherichia coli* could protect ampicillin-sensitive cohorts of other species, particularly species that could cause human disease. Both β -lactamase-secreting *E. coli* and, surprisingly, those engineered to retain it, allowed for survival of a large number of ampicillin-sensitive cohorts of *Salmonella enterica* serovar Typhimurium, including both laboratory and clinical isolates. The *Salmonella* survivors, however, remained sensitive to ampicillin when re-plated onto solid medium and there was no evidence of gene transfer. *Salmonella* survival did not even require direct physical contact with the resistant *E. coli*. The observed phenomenon appears to involve increased release of β -lactamase from the *E. coli* when present with *S. enterica*. Significantly, these findings imply that resistant *E. coli*, that are not themselves pathogenic, may be exploited, even when they are normally selfish with respect to other *E. coli*. Thus, *Salmonella* can gain protection against antibiotics from *E. coli* without gene transfer, a phenomenon not previously known. As a consequence, antibiotic-resistant *E. coli* can play a decisive role in the survival of a species that causes disease and may thereby interfere with successful treatment.

Keywords: *Salmonella enterica* serovar Typhimurium; β -lactamase; antibiotic susceptibility

1. INTRODUCTION

The emergence of antibiotic-resistant bacteria (e.g. *Shigella dysenteriae* (Sack *et al.* 1997); methicillin/oxacillin-resistant *Staphylococcus aureus* (Merlino *et al.* 2002); vancomycin-resistant enterococci (Rice 2001); extended-spectrum β -lactamases that confer resistance to cephalosporins and monobactams (Paterson & Bonomo 2005); also Martinez *et al.* 2007; Torres *et al.* 2007) has compromised effective treatment of infections. An extensive body of research has examined how new mechanisms of resistance evolve and how resistance, once it emerges, may spread throughout bacterial communities via promiscuous transfer of the genetic bases for such resistance (Hastings *et al.* 2004; and, for reviews, see Foster 1983; Smith 2001). More recently, we and others have explored an alternative mechanism of resistance, which depends on some members of the bacterial community acting as altruists and ‘sharing’ their resistance without transferring the genes responsible (Lenski & Hattingh 1986; Levin *et al.* 1997; Dugatkin *et al.* 2003;

Griffin *et al.* 2004; Dugatkin *et al.* 2005a,b; Clark *et al.* 2009). Such shared resistance can be conceptualized as something akin to a ‘public good’ in economics (Samuelson 1954; Brockhurst *et al.* 2008). One should note, however, that technically shared antibiotic resistance meets the ‘non-excludability’ condition, but probably not the ‘non-rivalness’ condition for a pure public good. We have demonstrated that *E. coli* that produces β -lactamase available to other bacteria allow sensitive cohorts of *E. coli* to survive in environments of otherwise lethal concentrations of antibiotics; similar findings of benefit have been observed with antibiotics or potentially toxic substances in biofilms (Karthikeyan *et al.* 2001; Donlan & Costerton 2002; Lynch & Robertson 2008) and in planktonic cultures (Lenski & Hattingh 1986; Rosensweig *et al.* 1994; Treves *et al.* 1998; Travisano 2001).

We have studied antibiotic resistance within populations of *E. coli* in the context of frequency-dependent selection. This theoretical framework, which has been extensively developed to understand the evolution of group-beneficial, public-good traits, has proven very useful for elucidating the dynamics of group-beneficial traits within microbial species. Our goal here is to expand this work done within species to the case of multi-species microbial communities, with a particular

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eye for how such studies can help us understand multi-species microbial communities that affect human health. In the long-run, we are interested in such questions as, can the public good provided by individuals from one microbial species benefit individuals from other species, and if so, what implications does this have for understanding the diversity of microbial species?

The group-beneficial trait we examine is antibiotic-resistance. Some bacteria naturally secrete the molecules that provide the mechanism for their resistance to antimicrobials. Gram-positive bacteria that display resistance to β -lactam antibiotics, such as penicillins (e.g. ampicillin; Foster 1983; Livermore 1997), often do so as a result of β -lactamases, typically high-affinity enzymes secreted into their environment. The degree of resistance conferred to cells in the medium (including both producers and others) is frequency-dependent: 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, otherwise antibiotic-susceptible cells may survive, and even thrive, in such settings without contributing to the resistance effort and paying a cost. Secretion of β -lactamases thus constitutes a public good (Brockhurst *et al.* 2008; Sheratt *et al.* 2009), which can be exploited by those that contribute neither to its production, nor to the benefit of the group as a whole. In contrast to examples of cooperation where production of the public goods increases with increasing resources, and thus, costs less (Brockhurst *et al.* 2008), here cooperation depends on the amount of β -lactamase needed for cells to withstand the antibiotics present in the microbial environment. We have explored this process by developing theoretical models (Dugatkin *et al.* 2003, 2005b, 2008), and by experimental work using near-isogenic bacteria, where the laboratory micro-environment can be controlled (Dugatkin *et al.* 2005a; Clark *et al.* 2009).

The evolution of resistance by pathogenic bacteria to the antibiotics used to treat serious infections poses a threat to the continued efficacy of such drugs in treatment. In this study, we examined the possibility that ampicillin-resistant *E. coli*, gram-negative bacteria that were engineered to secrete β -lactamase into their medium (Dugatkin *et al.* 2005a; Clark *et al.* 2009), might benefit ampicillin-sensitive cohorts of other species, especially species that could cause human disease. *Salmonella enterica* serovar Typhimurium is a causative agent of salmonellosis (Arrach *et al.* 2008). Most persons infected with this organism develop diarrhoea, fever, vomiting and abdominal cramps 12–72 h after infection. *Shigella flexneri* is another pathogenic enteric bacterium that accounts for about one-third of all cases of shigellosis in the USA. Shigellosis usually involves bloody diarrhoea, and, as with salmonellosis, fever and stomach cramps starting a day or two after they are exposed to the bacterium. People suffering with these infectious diseases may require hospitalization and shigellosis can lead to seizures, especially in young children. Here, we report that *S. enterica* serovar Typhimurium strains that are otherwise sensitive to ampicillin can survive in co-cultivation with ampicillin-resistant *E. coli*—even when *E. coli* cells produce β -lactamase but were not engineered to actively excrete this into the medium and do not permit antibiotic-sensitive *E. coli* strains to survive.

2. MATERIAL AND METHODS

(a) Host bacterial strains and plasmids

For these studies we used strains that could be distinguished visually on agar plates due to colour differences (e.g. in the presence of chromogenic substrates such as X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside)). A complete list of strains and plasmids is found in table S1 in the electronic supplementary material. *Escherichia coli* background strains KL99, 6925, Lu53 and Wyl, *S. enterica* serovar Typhimurium strain NI-101, and *Enterobacter aerogenes* ATCC 13048 are all blue on such media, whereas *E. coli* 5240 and the remaining *Salmonella* and *Shigella* strains are all white.

The plasmids used have been described previously (Clark *et al.* 2009). All plasmids are derived from pCR2.1 TOPO (Invitrogen, Carlsbad, CA, USA), 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 β -lactamase gene cloned into the multiple cloning site and placed under the control of the *lac* promoter of the vector. In the Alt strains, β -lactamase was engineered to be localized to the outer surface of the outer membrane where the effects of its action might be shared with other cells; the Alt strains contain the pSAR1 plasmid (Dugatkin *et al.* 2005a). In the Self strains, encoded on plasmid pSLAR1 (Dugatkin *et al.* 2005a), the β -lactamase was specifically engineered to be 'locked' in the periplasm. Hereafter in the text, strains bearing the pSAR1 and pSLAR1 plasmids will be referred to as Alt or Self, respectively. Plasmid pBR322 (Bolivar *et al.* 1977) is a standard ColE1-derivative bearing both a wild-type copy of the TEM-1 *bla* gene and a tetracycline-resistance gene.

(b) Measurement of β -lactamase activity, β -galactosidase activity and ampicillin concentrations

Beta-lactamase activity was measured using a spectrophotometric assay (Hedberg *et al.* 1995) with nitrocefin (Glaxo Research, Oxoid, UK) as the chromogenic cephalosporin substrate. The colour change from yellow to red when nitrocefin was cleaved by β -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 (Bradford 1976; Bio-Rad, Hercules, CA, USA). Ampicillin concentrations remaining in culture media were measured by the Folin-Ciocalteu method (Ahmad *et al.* 2004), using ampicillin standards in Davis Minimal Medium (DMM; Lenski *et al.* 1994) for the spectrophotometric assay. Activity for β -galactosidase was assayed according to the procedure recommended by Sambrook & Russell (2001).

(c) Conditions for growth and competitions

A dual-flask system (Bellco BIOTECHNOLOGY, Vineland, NJ, USA) was used in these experiments (Clark *et al.* 2009). The filter used between the two flasks was a Miracloth—0.2 μ m Supor-200 membrane (PALL) sandwich. This set-up allowed for dilution of waste products and maintenance of proper nutrient and drug concentrations. In competitions where physical contact among cells was permitted, both strains were diluted to the desired ratio (50 : 50) 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 (isopropylthio- β -D-galactoside, 0.16 mM), with or without ampicillin. All competitions done with both strains on the same side were run for at least 120 h, were done at least 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. Alternatively, for competitions in which direct physical contact was removed, one strain was inoculated on one side of the apparatus, and the other on the side normally used to replenish nutrients. In these cases, media was not exchanged during the course of the experiment, which normally was conducted for up to 96 h. In both set-ups, samples were taken every 12 h and analysed by measuring the absorbance at 600 nm (A_{600}) and by dilution plating, by spotting 40 μ l of 10^5 and 10^6 dilutions onto XI agar (containing X-gal (80 μ g ml $^{-1}$) + IPTG (0.16 mM)), AXI agar (XI + 100 μ g ampicillin per millilitre) and, for control experiments, KXI agar (XI + 50 μ g ml $^{-1}$ kanamycin). Three spots were counted for each dilution, comparing numbers of white versus blue colonies. From these measurements, colony forming units per millilitre were calculated for each strain for each growth culture.

(d) Statistical analyses

One-factor and univariate two-factor analysis of variance (ANOVA) were used to analyse initial growth and control experiments, as well as survival in competitions. As determined by Levene's test either Tukey HSD or Games-Howell *post hoc* multiple comparisons were performed. For time-course experiments, repeated-measures ANOVA was used for multiple comparisons with Sidak correction to reduce the possibility of Type I errors. Reject/fail-to-reject decisions for all null hypotheses were based on $\alpha = 0.05$. All routines were performed with PASW Statistics 17.2 (SPSS Inc., Chicago, IL, USA).

3. RESULTS

(a) In the absence of antibiotic, Salmonella strains outgrow E. coli strains

In nutrient-limiting liquid DMM (Lenski *et al.* 1994; Dugatkin *et al.* 2005a), the *Shigella* and *Salmonella* strains were compared for growth rates with those for the plasmid-bearing and plasmid-free background *E. coli* strains. In each case, *Salmonella* strains had more rapid rates of growth than *E. coli* or *Shigella*, either alone or in the same flasks, when in direct competitions with their *E. coli* counterparts (figure S1a in the electronic supplementary material). This was also true if the *E. coli* and either *Shigella* or *Salmonella* were inoculated into opposite sides of a dual-flask set-up used in competition experiments (Clark *et al.* 2009). On the other hand, all the *Shigella* and *Salmonella* strains in this study were unable to grow in ampicillin concentrations at or above 100 μ g ml $^{-1}$, either in liquid DMM (figure S1b in the electronic supplementary material) or on solid DMM agar or rich Luria-Bertani (LB) agar. To further assess these differences in growth, the relative susceptibilities (minimum inhibitory concentrations (MICs)) to ampicillin of *S. flexneri* ATCC 12022, *S. enterica* serovar Typhimurium ATCC strain 14028, and the background *E. coli* strains 5240 and 6925 were determined after growth in DMM broth with subsequent spotting onto LB agar plates

containing different concentrations of ampicillin. From these assessments (see table S2 in the electronic supplementary material), we found that the *S. flexneri* ATCC 12022 was by far the most susceptible to ampicillin (MIC 10 μ g ml $^{-1}$), whereas the plasmid-free *E. coli* strains displayed intermediate susceptibility (MIC 25–50 μ g ml $^{-1}$). Interestingly, the *S. enterica* serovar Typhimurium ATCC strains (14028, NI-1), while susceptible at 50 μ g ampicillin per millilitre, persisted at low levels (about 1% of that seen, for example, on 25 μ g ampicillin per millilitre) even on agar containing 100 μ g ampicillin per millilitre. All plasmid-bearing *E. coli* in this study had MICs greater than 400 μ g ampicillin per millilitre.

(b) Otherwise antibiotic-sensitive Salmonella cells survive when grown together with E. coli engineered to secrete β -lactamase

We have previously shown that ampicillin-sensitive *E. coli*, grown with near-isogenic ampicillin-resistant altruist *E. coli* bearing the pSAR1 plasmid, were able to survive normally lethal concentrations of the drug (Dugatkin *et al.* 2005a; Clark *et al.* 2009). This was again observed here, where sensitive *E. coli* 5240 survived at approximately 3 per cent when present with the *E. coli* KL99Alt (Dugatkin *et al.* 2005a; Clark *et al.* 2009; figure 1a) or 6925Alt (figure 1a). No such survivors were observed in identical competitions between 5240 and KL99Self (figure 1b). To explore the possibility that such *E. coli* cells bearing pSAR1 could similarly protect sensitive cohorts of different species, *S. flexneri* ATCC 12022 and *S. enterica* serovar Typhimurium strains were each competed with KL99Alt. No *S. flexneri* ATCC 12022 survivors were observed in such competitions, at either 50 or 100 μ g ampicillin per millilitre. As seen in figure 1a, for competitions in 100 μ g ampicillin per millilitre, at 12 h sampling, the colony forming units per millilitre of *S. enterica* serovar Typhimurium ATCC strain 14028 dropped below 2.5×10^6 . However, survivors of this strain were detected after 24–36 h of growth in the competition flasks at either concentration of ampicillin; their numbers increased throughout the course of competitions, until they represented roughly 80 per cent of survivors on LB agar containing X-gal and IPTG (XI) plates. However, when bacteria from these same competitions were plated directly onto XI agar plates containing 100 μ g ampicillin per millilitre (AXI agar plates), no *S. enterica* serovar Typhimurium colonies grew. In addition, all *S. enterica* serovar Typhimurium survivors that appeared on XI agar plates were ampicillin-sensitive (i.e. they failed to grow when re-streaked onto AXI agar plates). As controls, competitions were also conducted between ampicillin-sensitive *E. coli* plasmid-free background strains KL99 or 6925, and these similarly failed to yield either *S. enterica* serovar Typhimurium or *S. flexneri* ATCC 12022 survivors in the presence of 100 μ g ampicillin per millilitre of DMM broth (not shown; statistically significant differences in per cent survivors, representing *p*-values < 0.01 , from Games-Howell *post hoc* analyses). Moreover, *S. enterica* serovar Typhimurium 14028 had significantly greater fitness than *S. flexneri* strain 12022 or plasmid-free *E. coli* strains competed with the ampicillin-resistant *E. coli*.

Figure 1. Otherwise selfish ampicillin-resistant *E. coli* support the survival of *Salmonella*. *Salmonella enterica* serovar Typhimurium 14028 or *S. flexneri* strain 12022 were competed against (a) Altruist *E. coli* strains or (b) Selfish *E. coli* strains in 100 µg ampicillin per millilitre DMM. Additional competitions with *Salmonella* were conducted (c) at 50:50 initial starting proportions of strains in 100 µg ampicillin per millilitre DMM liquid medium; or (d) in DMM liquid medium without ampicillin. Presented are the results of three independent replicates, with the mean per cent ampicillin-sensitive survivors on XI shown, along with standard error bars. Those samples whose cfu ml⁻¹ were less than 2.5×10^6 are represented as 0 in this graph. In each case, the plasmid-free ampicillin-sensitive strain is indicated and, in parentheses, its plasmid-bearing ampicillin-resistant competitor (as, e.g. vKL99Alt). 5240 Free, 5240 without plasmid; *Salmonella*, *S. enterica* serovar Typhimurium ATCC 14028; *Shigella*, *S. flexneri* strain 12022; *Shigella*(vKL99Self50), the *S. flexneri* strain 12022 was competed with *E. coli* KL99Self at 50 µg ampicillin per millilitre DMM. (a) Dark blue, 5240Free(vKL99Alt); yellow, *Salmonella*(vKL99Alt); purple, *Salmonella*(v6925Alt); light blue, *Shigella*(vKL99Alt). (b) Dark blue, 5240Free(vKL99Self); yellow, *Salmonella*(vKL99Self); purple, *Salmonella*(v6925Self); green, *Shigella*(vKL99Self); light blue, *Shigella*(vKL99Self50). (c) Dark blue, KL99Alt; yellow, KL99Self; purple, 6925Self; green, Lu53pBR322; light blue, Wyl. (d) Purple, 6925Self; green, Lu53pBR322; light blue, Wyl; dark green, 6925Free.

(c) Other ampicillin-resistant *E. coli* also allow sensitive *Salmonella* cells to survive

The *E. coli* strain KL99Self (Dugatkin *et al.* 2005a; Clark *et al.* 2009) is resistant to ampicillin, but does not share resistance with sensitive *E. coli* (Clark *et al.* 2009). The survival of sensitive *E. coli* in ampicillin when competed with KL99Alt was approximately 3 per cent (figure 1a), compared with no observed survivors in competitions of the sensitive *E. coli* with the Selfish *E. coli* (figure 1b). This difference was nearly statistically significant ($p = 0.093$).

To our surprise, this Selfish strain also allowed otherwise ampicillin-sensitive *S. enterica* serovar Typhimurium to grow (figure 1b), at a rate comparable to that provided by the KL99Alt and this benefit was observed even when competitions were in the presence of starting concentrations of 200 µg ampicillin per millilitre of DMM. At 200 µg ampicillin per millilitre DMM broth, at 36 h, the *S. enterica*-sensitive cells were roughly 60 per cent of the survivors on XI agar, and this increased to 68.6 per cent by 60 h (not shown). When the starting ratios of *E. coli* to *S. enterica* serovar Typhimurium were 75:25, the proportion of *S. enterica* serovar Typhimurium became 64.4 per cent by 36 h, and this proportion continued to rise until the *S. enterica* serovar Typhimurium was 75.8 per cent of the survivors plated onto XI, a level comparable to what was observed with starting ratios of 50:50 *E. coli* to *S. enterica* serovar Typhimurium (not shown). With starting ratios of 10:90, although at 24 h *S. enterica*

serovar Typhimurium-sensitive cells yielded less than 2.5×10^6 cfu ml⁻¹, by 48 h, they had become 75 per cent of the survivors on XI agar plates (not shown).

Since *E. coli* KL99 is an Hfr strain, we tested the possibility that survival of *S. enterica* serovar Typhimurium survivors depended on the transfer (either permanent or transient) of the *bla* gene from the *E. coli* strain. As mentioned earlier, all *S. enterica* serovar Typhimurium survivors obtained from XI plates remained sensitive to ampicillin, and none examined contained plasmids. However, to further rule out gene transfer from the KL99 background as explanation for our findings, we repeated the competition experiments using strains that lack conjugative transfer ability (i.e. 6925Self and Lu53(pBR322); the latter strain bears the wild-type TEM-1 *bla* gene on the classic plasmid vector, pBR322). Although at 24 h, co-cultivation with the latter strain yielded only about 5 per cent of the otherwise sensitive *S. enterica* serovar Typhimurium cells observed with 6925Self; by 48 h, the proportions of *S. enterica* cheaters produced by both *E. coli* strains (figure 1c) were comparable, although Lu53(pBR322) still yielded a significantly lower proportion. Moreover, similar numbers were obtained when samples were plated onto XI or DMM + XI, or onto AXI versus DMM + AXI. In these experiments, an equilibrium was established, whereby the sensitive *S. enterica* failed to exclude their ampicillin-resistant *E. coli* benefactors. Similar results were obtained at both 100 (figure 1c) and 200 µg ampicillin per millilitre of DMM (not shown). On the

other hand, when competitions were conducted in LB broth (i.e. rich medium) containing 100 µg ampicillin per millilitre, cultures grew to much higher densities and at 48 h essentially 100 per cent of survivors observed by plating 10^5 dilutions onto XI agar were *S. enterica* serovar Typhimurium (not shown).

(d) Survival of otherwise ampicillin-sensitive cells is not limited to one *Salmonella* strain and does not require plasmid-borne *bla*

In order to rule out the possibility that the laboratory strain of *S. enterica* serovar Typhimurium used might be a unique variant or mutant, the above experiments were repeated with several additional isolates, including another source of ATCC 14028, and two *S. enterica* serovar Typhimurium clinical isolates, NI-1 and NI-101. Competitions between each of these isolates and a Selfish *E. coli* (6925Self or 5240Self) yielded results comparable to those already described with our original laboratory source of *S. enterica* serovar Typhimurium ATCC 14028 (figure S2 in the electronic supplementary material).

From the control competitions with plasmid-free, ampicillin-sensitive *E. coli*, it was clear that survival of otherwise sensitive *S. enterica* serovar Typhimurium cells required the presence of a β -lactamase-producing *E. coli* strain. Neither sensitive *E. coli*, nor a β -lactamase-negative, ampicillin-resistant *E. aerogenes* strain (figure 2a) yielded *S. enterica* serovar Typhimurium survivors, which were otherwise sensitive to ampicillin in competition experiments. To examine whether such cell production was dependent on the genetic/physical location of the *bla* gene in the resistant *E. coli*, competitions were conducted between an *E. coli* strain, Wyl, in which the *bla* gene was integrated via recombination into the chromosome (Chait *et al.* 2007). As seen in figure 1, *Salmonella* cells that were otherwise sensitive to ampicillin also survived when co-cultivated with this strain, albeit at significantly less proportions ($p < 0.01$) than with the plasmid-borne *bla* in KL99Alt, KL99Self or 6925Self. Moreover, in each case, when no ampicillin was present, the *S. enterica* ATCC 14028 strain grew to over 80 per cent of the mixed population within 24 h (figure 1d).

(e) Physical contact between ampicillin-resistant *E. coli* and susceptible *Salmonella* is not required for protection

As a preliminary assessment of additional requirements for *S. enterica* serovar Typhimurium survival, competitions between ampicillin-resistant *E. coli* and susceptible *S. enterica* serovar Typhimurium isolates were conducted, with the competitors inoculated into opposite sides of a dual-flask competition apparatus that allowed exchange of low-molecular weight compounds, including proteins and enzymes, but prevented physical contact of cells on the opposite sides of a membrane (Clark *et al.* 2009). We determined that neither bacterial strain was able to cross the membrane, and most common coliphages also fail to cross such membranes (Rapp *et al.* 1992). Although we could not rule out smaller phages crossing the membrane, in preliminary phage titre assays, we did not observe evidence that lytic phage capable of

Figure 2. No physical contact is required for the survival of *Salmonella* when co-cultivated with ampicillin-resistant *E. coli*. *Salmonella enterica* serovar Typhimurium ATCC 14028 was grown in competition with 6925Self or with *E. aerogenes* ATCC 13048 in the presence of 100 µg ampicillin per millilitre DMM. Presented are the results of three independent replicates with (a) the mean per cent of *Salmonella* colony forming units per millilitre on XI plates shown or (b) the absorbance at 600 nm of the *Salmonella* side of dual-flasks. Those samples whose cfu ml⁻¹ were less than 2.5×10^6 are represented as 0 in this graph. Standard error bars are shown. (a) Strains were inoculated into the same side of a dual-flask apparatus; v6925Self, competition of *Salmonella* against *E. coli* 6925Self; vEnterero, competition of *Salmonella* against *E. aerogenes* ATCC 13048. Diamonds, *Salmonella* v6925Self; triangles, *Salmonella* vEnterero. (b) Strains were inoculated into opposite sides of a dual-flask apparatus. vEnterero, competition with one flask containing *E. aerogenes* and the other side of dual-flask apparatus containing *S. enterica* serovar Typhimurium ATCC 14028. Diamonds, *Salmonella* v6925Self; triangles, *Salmonella* vEnterero. (c) 6925Free (plasmid-free, ampicillin-sensitive *E. coli*) was inoculated into the opposite side of a dual-flask apparatus either: alone, opposite 6925Self and *S. enterica* serovar Typhimurium ATCC 14028; or, together 'with *Salmonella*' strain 14028, while Selfish *E. coli* 6925Self was inoculated into the opposite side of the dual-flask set-up.

producing plaques were present in any of the cultures (data not shown).

When *S. enterica* serovar Typhimurium strains were competed without contact against KL99Self or 6925Self in the presence of ampicillin, *S. enterica* serovar Typhimurium cells grew at proportions comparable to what was observed when the respective strains were competed

together in the same flask (and fresh media was exchanged at 12 h intervals). Thus, *S. enterica* serovar Typhimurium growth does not depend on direct contact with the resistant *E. coli* (figure 2b).

We additionally sought to determine whether the interaction between resistant *E. coli* strains and ampicillin-susceptible *S. enterica* serovar Typhimurium isolates could benefit sensitive *E. coli* too, resulting in survival of *E. coli* cells that were otherwise sensitive to ampicillin. In ampicillin, when *E. coli* 6925Self was grown on the same side of the dual-flask set-up with *S. enterica* serovar Typhimurium 14028, plasmid-free *E. coli* 6925 cells survived when inoculated into the opposite side of the set-up, containing the same concentration of antibiotic (figure 2c). After 48 h, *E. coli* 6925 cells grew when plated onto XI agar (at frequencies about 30% of those seen for *S. enterica* serovar Typhimurium 14028 (figure 2c); no *E. coli* 6925 colonies appeared on AXI). When the plasmid-free *E. coli* 6925 and *S. enterica* serovar Typhimurium 14028 were inoculated onto the same side of the dual-flask apparatus and 6925Self was inoculated alone into the opposite side (figure 2c), the number of plasmid-free *E. coli* 6925 survivors only reached about one-seventh the level observed in the previous experiment (i.e. when the plasmid-free *E. coli* 6925 was in the opposite side of the set-up and *E. coli* 6925Self grew together with *S. enterica* serovar Typhimurium 14028).

(f) Co-cultivation of ampicillin-resistant *E. coli* with susceptible *Salmonella* leads to free β -lactamase in the medium

Levels of β -lactamase were measured in medium collected from *E. coli* and *S. enterica* serovar Typhimurium cells grown in competitions. Levels were determined for the spent media and for sonic extracts of collected cells, for strains grown individually, strains grown in the same flasks for competitions and strains grown on opposite sides of flasks in the dual-flask set-up. β -Lactamase activity was found in supernatants of ampicillin-resistant *E. coli* grown with *S. enterica*. Such activity was also observed on the *S. enterica* side of the dual-flask apparatus. No β -lactamase activity was detected in supernatants where ampicillin-sensitive *E. coli* or *S. enterica* serovar Typhimurium had grown alone. In the absence of ampicillin, for the most part, a larger proportion of β -lactamase activity was found in the supernatant fraction of cultures. On the other hand, in most cases the total levels of β -lactamase activity were higher when ampicillin-resistant *E. coli* were cultured together with *S. enterica* in the presence of ampicillin (versus the same comparison in the absence of ampicillin; figure 3). At the same time, the larger proportion of total activity was predominantly in sonic extracts of cells. This may, in part, reflect selection for higher proportions of β -lactamase-producing *E. coli* in such cultures (due to the presence of ampicillin). As an indicator of cell lysis, we also assayed for activity of a large cytoplasmic enzyme, β -galactosidase. As seen in figure 3b, although the bulk of the enzyme activity remained within the cells, in some cases this enzyme was released at substantial proportions, indicating cell lysis. In the case of the Wyl strain in competitions with *S. enterica*, the majority of the β -galactosidase was found in the supernatant.

(g) Media recovered from competitions does not inhibit the growth of newly inoculated strains

Growth media from competitions and from strains grown alone were recovered by centrifugation and subsequent filtration through 0.2 μ m filters for sterilization. In this way we sought to characterize properties of the respective media vis-à-vis effects on growth of strains newly inoculated therein. Little or no growth was observed for the *E. coli* strains 6925 and 6925Self or *S. enterica* serovar Typhimurium ATCC 14028 when inoculated into any of these media. However, when additional nutrients (final concentrations of dextrose (0.006%), MgSO_4 (1 mM), thiamine (0.0005%), Casamino acids (0.004%) and IPTG (0.16 mM)) were added, each of these strains grew in the media (see figure S3 and table S3 in the electronic supplementary material). If, in addition to nutrients, ampicillin was added to a predicted final concentration (ignoring any residual ampicillin already present in the supernatants) of 100 $\mu\text{g ml}^{-1}$, the growth rates and final cell densities of each culture depended on the source of the supernatant. If obtained from cultures originally containing ampicillin-resistant *E. coli*, growth was similar to what was observed without the addition of drug, not only for the ampicillin-resistant *E. coli* and sensitive *S. enterica* serovar Typhimurium ATCC 14028, but even for the ampicillin-sensitive strains such as *E. coli* 6925 and *S. flexneri* ATCC 12022 (figure S3 in the electronic supplementary material). If the supernatant was originally obtained from cultures of *S. enterica* serovar Typhimurium ATCC 14028 grown alone, then addition of nutrients and ampicillin only allowed the growth of the ampicillin-resistant 6925Self, while the other ampicillin-sensitive strains (plasmid-free 6925 and *S. enterica* serovar Typhimurium ATCC 14028, or *S. flexneri* ATCC 12022) failed to grow (figure S3, top panel in the electronic supplementary material).

4. DISCUSSION

In this report, we have extended the previous studies that used a bacterial model for the production of public goods, leading to survival of otherwise antibiotic-susceptible cohorts (Dugatkin *et al.* 2005a; Clark *et al.* 2009). Here, we sought to explore the possibility that *E. coli* engineered to release β -lactamase could lead to the survival of otherwise ampicillin-sensitive cells in a setting that more realistically models a natural setting, where they normally have to compete not only with other *E. coli*, but with a wide variety of gut flora. Among these are organisms that cause gastrointestinal disease. *Salmonella enterica* serovar Typhimurium is known to cause gastrointestinal disease and this requires that cells of this species establish themselves in an environment already colonized with other bacteria. *Salmonella enterica* serovar Typhimurium are apparently highly effective at such colonization (Esteves *et al.* 2005). What is more, the would-be pathogens are more effective in binding to epithelial cells, such as those that line the gut and they can displace the *E. coli* that have already attached (Esteves *et al.* 2005). Thus, when introduced into the gut ecosystem, *S. enterica* serovar Typhimurium would appear to be somewhat at an advantage in competitions with *E. coli*. On the other hand, antibiotic-susceptible *S. enterica* serovar

Figure 3. β -Lactamase present in competitions is found in both pelleted cells and in the collected growth medium. Competitions (50:50 initial starting proportions of strains or with each strain alone) were conducted at 100 μ g ampicillin per millilitre DMM liquid medium or without antibiotic. The values presented here represent samples collected after 48 h growth. (a) β -Lactamase activity expressed as absorbance at 486 nm (A_{486}) per minute per microgram of protein. (b) Activity of β -galactosidase. *Salmonella enterica* serovar Typhimurium ATCC 14028 is unable to produce this enzyme, hence the absence of activity from it and the reduction in specific activity when this organism dominates the cultures. Dark bars—supernatant, filter-sterilized supernatant from competition; light bars—sonic extract, sonic extract of collected cell pellet from competition.

Typhimurium (and other species) should be selected against and eliminated in the presence of sufficiently high levels of drug, such as ampicillin, while ampicillin-resistant cells should survive and have increased fitness.

In the current experiments, ampicillin-resistant *E. coli* survived, but so did otherwise ampicillin-susceptible *S. enterica* serovar Typhimurium. These latter cells did not acquire genetically based ampicillin-resistance but rather, remaining sensitive, they survived the conditions of growth. Not only did these cells survive, but they actually thrived under these conditions. When ampicillin was present, an equilibrium developed, whereby the sensitive *S. enterica* grew to large proportions of the culture, but failed to exclude their ampicillin-resistant *E. coli* benefactors. This equilibrium took time to establish itself. The *S. enterica* serovar Typhimurium survived when grown with *E. coli* that had been engineered to export β -lactamase

to the outer membrane; in contrast, ampicillin-sensitive *S. flexneri* were unable to survive under similar conditions. This is probably due to a much greater intrinsic susceptibility to ampicillin of the *S. flexneri* strains used in this study compared with either the *S. enterica* serovar Typhimurium or the ampicillin-sensitive *E. coli* (table S2 in the electronic supplementary material). On the other hand, apparently the sole requirement for *S. enterica* serovar Typhimurium survivors was that their *E. coli* benefactor must themselves produce β -lactamase, which gets released to higher levels when *E. coli* grow in the presence of the *S. enterica* serovar Typhimurium. This is clearly an example where an extracellular product or public good can fortuitously benefit others (Sheratt *et al.* 2009). The results of the present study are of a kind, up to a point, with a variety of reports where resistance to antibiotics is observed but is not heritable (for an overview,

see Levin 2004; also, see Barclay *et al.* 1992; Gradel ski *et al.* 2001). However, a difference here is the added interaction between antibiotic-resistant cells of one species and the sensitive beneficiaries of another. When not constrained by ampicillin, the aggressive growth of the *S. enterica* serovar Typhimurium ATCC 14028 was compatible with the findings of Esteves *et al.* (2005), who found that *S. enterica* had a higher fitness (as measured by overall cell densities achieved) than *E. coli* when these species were grown together. However, the findings of Esteves *et al.* (2005) do not provide the mechanism exploited by *S. enterica* serovar Typhimurium ATCC 14028 to survive when inoculated into lethal ampicillin concentrations.

One possible explanation for the survival of *S. enterica* serovar Typhimurium ATCC 14028 in the presence of ampicillin in our study is the transient expression in this organism of an otherwise cryptic ampicillin-resistance gene. However, if this is the case, it is only effective when ampicillin-resistant *E. coli* are also present.

It is also possible that *S. enterica* serovar Typhimurium produces a substance or entity that increases leakiness of *E. coli*. The media spent from cultures that contained *S. enterica* serovar Typhimurium did not seem to interfere with the growth of *E. coli*. Thus, at present, we have no data that indicate either the presence of a phage or other products released by the *S. enterica* serovar Typhimurium, which inhibit the growth of *E. coli* or likely increase its lysis.

For competitions in ampicillin-containing media, the observation that within the first 12–24 h, *S. enterica* numbers drop from 50 per cent to nearly 0 per cent (data from figures S1 and S2 in the electronic supplementary material) and then rebound to levels of 60–90%, is significant. It suggests that after 12–24 h of competition with *bla*-containing *E. coli*, there has been a change in the environment experienced by the competing bacteria that allows cells to survive and grow, even as ampicillin is replenished. Further, the growth experiments with media recovered from competitions suggest that, by 24 h, survival of cells in ampicillin is no longer dependent on the frequency of β -lactamase producers. Thus, a simpler explanation for the observed survival of *S. enterica* serovar Typhimurium may be related to the release and/or production of β -lactamase by the resistant *E. coli*.

We observed that β -lactamase-producing *E. coli* had greater net amounts of enzyme in ampicillin-containing media and greater total enzyme activity was retained within the cells (figure 3). This observation may be due to increased selection for cells that produce the enzyme in sufficiently high amounts to survive under these conditions. Still, large quantities of β -lactamase were also detected in the supernatants from competitions of such strains with *S. enterica*. The detection of activity for a large cytoplasmic enzyme (i.e. β -galactosidase) in such supernatants suggests that some cell lysis has occurred under these conditions. At the same time, co-culturing with *S. enterica* serovar Typhimurium was associated with increased β -lactamase levels and/or leakiness of the ampicillin-resistant *E. coli* cells in the presence of ampicillin. It is known that a variety of growing Gram-negative bacteria release membrane blebs (Katsui *et al.* 1982; Kadurugamuwa & Beveridge 1997; Li *et al.* 1998; Beveridge 1999; Post *et al.* 2005). Such blebs are associated with abnormal in-growth of the outer membrane,

and they typically appear around the septa during cell division. They often contain phospholipids and a range of periplasmic enzymes, including β -lactamase (Ciofu *et al.* 2000), which are eventually further released into the medium (Kadurugamuwa & Beveridge 1997; Li *et al.* 1998). Moreover, release of blebs increases under conditions of stress, such as antibiotic exposure, serum containing complement or nutrient deprivation (Knox *et al.* 1966; MacDonald & Beveridge 2002). Perhaps, the individual and combined stresses of nutrient deprivation, antibiotic exposure and competition with a robust and vigorously growing competitor (i.e. *S. enterica* serovar Typhimurium) increasingly lead β -lactamase-producing *E. coli* to substantially release their enzymes into the media, to the benefit of ampicillin-sensitive cells.

It has recently been shown that antibiotic treatment has dramatic effects on the composition and diversity of the gut microbiota (Dethlefsen *et al.* 2008). Moreover, despite the apparent resilience of the gut community, long-lasting changes in composition often do occur (Dethlefsen *et al.* 2008). The results presented here suggest that the production of substances that break down the antibiotics in one species of gut microbiota (e.g. *E. coli*) has implications not just for that species, but other species in the community in which it resides. The studies described here are significant, for they show that the β -lactamase produced by strains of *E. coli* may be associated with maintenance of diversity of bacteria microbiota communities, the evolution of antibiotic resistance, and survival of potential pathogens in otherwise lethal concentrations of drug. It has already been demonstrated that 'cheats' may exploit situations where wild-type cells cooperate to enhance virulence in clinical settings (Köhler *et al.* 2009). Our results demonstrate clearly that non-pathogenic antibiotic-resistant bacteria may protect otherwise susceptible pathogens by a mechanism that does not involve gene transfer. Evolution of such transient survival mechanisms may thereby hinder therapeutic use of antibiotics, and should be considered in devising effective treatment strategies.

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