



Cooperative resistance varies among β -lactamases in *E. coli*, with some enabling cross-protection and sustained extracellular activity



Qinqin Wang^{1,2}, Shaodong Wei² & Jonas Stenløkke Madsen  

β -lactamases confer bacteria resistance to β -lactam antibiotics, and interestingly, this protective effect can extend to neighboring susceptible cells. However, knowledge of this cooperative resistance remains limited. Here, we investigated the underlying factors of cooperative resistance to assess commonalities and differences among the highly diverse group of β -lactamases. We first analyzed β -lactamase genes from 2637 *Escherichia coli* genomes, followed by experimental characterization of seven prevalent β -lactamase genes. Larger plasmids, particularly conjugative ones, commonly encoded β -lactamases. All seven genes had strong wildtype promoters, and plasmid-based expression rescued more susceptible bacteria than chromosomal expression. Cooperative resistance positively correlated with β -lactamase activity and minimal inhibitory concentrations. Cross-protection could be established between different β -lactamase producers, challenging the effectiveness of therapies combining β -lactams. Extracellular activity varied among β -lactamases and, when high, resulted in a legacy resistance effect in the environment. These findings advance our understanding of β -lactam resistance and highlight important implications for antibiotic treatment strategies.

Antibiotic resistance has been regarded as one of the major threats to human health for decades, and the number of deaths due to antibiotic-resistant infections is on the rise^{1,2}. β -lactam resistance is the most common among bacteria, and its emergence and spread seem inseparable from the wide use of β -lactam antibiotics³. β -lactam resistance is commonly acquired through the production of β -lactamases, which hydrolyze the β -lactam ring⁴. Intriguingly, β -lactamases have been shown to act as public goods that can rescue neighboring sensitive cells^{5,6}, a phenomenon referred to as cooperative antibiotic resistance⁷. Sensitive bacteria rescued by cooperative antibiotic resistance can directly expand the potential recipient pool of horizontally acquired antibiotic resistance genes, which increases the risk and scope of the spread of resistance⁸. It is known from gut-microbiome studies that β -lactamases can protect sensitive bacteria *in vivo*, thereby sustaining diversity^{9,10}, and it has been argued that cooperative resistance likely changes the trajectory of resistance evolution during polymicrobial infections¹¹. Cooperative β -lactamase resistance has also been shown *in vivo* to increase the failure rates at eradicating bacterial infections, thus, potentiating a major

public health problem with the rapid proliferation of β -lactamase-producing pathogens¹².

The common opportunistic pathogen *Escherichia coli* exhibits β -lactam resistance mainly associated with the production of β -lactamases¹³ and often harbors multiple plasmids that encode antibiotic resistance genes, leading to multidrug resistance¹⁴. It is now well established that conjugative plasmids play a pivotal role in the spread of antibiotic resistance genes¹⁵. Bacteria not only acquire antibiotic resistance through conjugational transfer and thereby improve their fitness under antibiotic treatment, but also promote the evolution of antibiotic resistance through non-conjugative plasmids carrying adaptive genes^{16,17}. Moreover, co-evolution between plasmids and host bacteria, plasmid transmission dynamics, and fitness costs have all been shown to be relevant to the development of antibiotic resistance^{16,18,19}. Furthermore, plasmids are thought to have a potential role in facilitating bacterial cooperation²⁰.

Insights into the cooperative resistance potential of β -lactamases from different classes may lead to the development of new interventions aimed at

¹Section of Microbiology, Department of Biology, University of Copenhagen, Copenhagen, Denmark. ²National Food Institute, Technical University of Denmark, Kgs Lyngby, Denmark.  e-mail: jsmadsen@bio.ku.dk

minimizing interbacterial resistance and the spread of resistance genes in pathogens. Most current studies focus on the class A β -lactamases, and a comprehensive comparison and evaluation of the cooperative potential of all four different classes of β -lactamases are lacking. In this work, we studied underliers of cooperative resistance to assess commonalities and differences across β -lactamases. Our goal was to investigate (1) if cooperative resistance is an innate property of β -lactamases regardless of class, (2) if higher expression and activity of β -lactamases generally enhances cooperative resistance, (3) if horizontal transfer of β -lactamases can occur during β -lactam exposure due to rescue of sensitive cells, and (4) if extracellular activity of β -lactamases is especially important for efficient cooperative resistance.

We investigated the distribution of β -lactamase in *E. coli* in silico and further experimentally tested seven β -lactamases from four β -lactamases classes. Our experimental data suggest that cooperative resistance is universal among β -lactamases, with varying rescue levels for susceptible cells. Efficient plasmid transfer was observed even in the presence of β -lactams. Elevated β -lactamase expression associated with plasmids increased cooperative resistance and facilitated cross-resistance. We find that both intracellular and extracellular β -lactamase activity were strong underliers of cooperative resistance; however, high extracellular activity was essential for imposing environmental resistance legacy effects. These results provide more insights into the association between genotype, gene expression, gene copies, horizontal gene transfer, and cooperative resistance.

Results

β -lactamase genes are widespread among *Escherichia coli* and commonly encoded in conjugative plasmids

Based on available complete and closed DNA sequenced genomes of *Escherichia coli* isolates ($n = 2637$), β -lactamase genes were identified using the Comprehensive Antibiotic Resistance Database²¹. The majority of *E. coli* ($n = 2566$) carried β -lactamase genes (*bla* genes), and only very few ($n = 71$) did not (Fig. 1A). Based on the *bla* genes classification, class C represented the largest proportion (50.3%), followed by the class A (38.32%), D (6.7%), and B (4.7%) (Fig. 1B). The main reason for such a large proportion of class C genes was *bla_{ampC}* and *bla_{ampC1}*, which are common in *E. coli* chromosomes but are typically not expressed due to a lack of the regulatory gene *ampR*²² (Supplementary Fig. 1A). Moreover, plasmids carried a much bigger proportion of *bla* genes compared to chromosomes (Fig. 1B and Supplementary Fig. 2). It is noteworthy that the *bla* genes of all four classes most often were associated with conjugative plasmids over non-conjugative plasmids (Fig. 1B). The highest number of *bla* genes found encoded on a chromosome was 12 (Fig. 1C). This was fewer than the number of *bla* genes found in individual plasmids, one plasmid even encoded 35 *bla* genes. The number of *bla* genes encoded in plasmids was positively correlated with the size of plasmids ($\rho = 0.37$; $P < 0.001$, Spearman correlation). In contrast,

this was not the case for chromosomes ($\rho = 0.02$; $P = 0.24$, Spearman correlation) (Fig. 1C). Overall, our results underscore the ubiquity of *bla* genes in the *E. coli* genomes and that there is a prevalence of β -lactamase genes in plasmids, especially conjugative ones, compared to chromosomes.

Bla_{ampC}, Bla_{TEM-1}, Bla_{CTX-M-15}, Bla_{CMY-2}, Bla_{NDM-5}, Bla_{KPC-2}, and Bla_{OXA-181}, representing all four β -lactamase classes, are all cooperative in cocultures and transcribed from strong wild-type promoters

In view of the results presented in Fig. 1, seven *bla* genes with high abundances and representing the four classes were selected for further experimental studies. These were *bla_{ampC}*, *bla_{TEM-1}*, *bla_{CTX-M-15}*, *bla_{CMY-2}*, *bla_{NDM-5}*, *bla_{KPC-2}*, and *bla_{OXA-181}* (Supplementary Fig. 1). The seven different *bla* genes were cloned from the native plasmids, together with gene *sfGFP* (encoding green fluorescent protein), into *E. coli* MG1655, either into the chromosome, a conjugative plasmid (pJK5), or a non-conjugative mutant of the plasmid (pJK5_{NC})²³. Thus, three distinct β -lactamase producers were used for each *bla* gene, namely the chromosomal producer (termed as “P_{chr}-gene”, such as P_{chr}-*bla_{ampC}*, P_{chr}-*bla_{CTX-M-15}*, etc.), the conjugative plasmid producer (termed as “P_{conj}-gene”, such as P_{conj}-*bla_{ampC}*, P_{conj}-*bla_{TEM-1}*, etc.), and the non-conjugative plasmid producer (termed as “P_{non-conj}-gene”, such as P_{non-conj}-*bla_{ampC}*, P_{non-conj}-*bla_{TEM-1}*, etc.). The different *bla* genes were initially kept in association with their wild-type promoters (P_{WT}); however, to investigate and compare the different *bla* genes in a more direct manner, the different wild-type promoters were also exchanged with the P_{A1-O4/O3} promoter. P_{A1-O4/O3} is known to be strong when the lactose operon is not repressed²⁴. These producers were named accordingly, chromosome producers (termed as “P_{chr}-P_{A1}-gene”, such as P_{chr}-P_{A1}-*bla_{ampC}*, P_{chr}-P_{A1}-*bla_{TEM-1}*, etc.), conjugative plasmid producers (termed as “P_{conj}-P_{A1}-gene”, such as P_{conj}-P_{A1}-*bla_{ampC}*, P_{conj}-P_{A1}-*bla_{TEM-1}*, etc.), and non-conjugative plasmid producers (termed as “P_{non-conj}-P_{A1}-gene”, such as P_{non-conj}-P_{A1}-*bla_{ampC}*, P_{non-conj}-P_{A1}-*bla_{TEM-1}*, etc.).

The minimum inhibitory concentrations (MIC) of all strains were determined for penicillin (PEN) and cephalothin (CEF, first-generation cephalosporin), to assess differences between the β -lactamase types and the promoters (Supplementary Table 1, Fig. 2A for PEN and Supplementary Fig. 3A for CEF). With the exception of *bla_{TEM-1}*, the MIC values of the other six *bla* genes with P_{WT} against the PEN were all higher than when transcribed from the P_{A1-O4/O3} promoter (Fig. 2A). A similar trend was observed for MIC values of CEF, except that *bla_{TEM-1}* and *bla_{OXA-181}* had lower MICs toward CEF (Supplementary Fig. 3A). The relative difference of strength between the P_{A1-O4/O3} and the different P_{WT} promoters was approximated by calculating the ratio between the MICs in PEN (MIC_{PEN} [P_{WT}]/MIC_{PEN} [P_{A1-O4/O3}]) (Supplementary Fig. 4A). Our analysis showed that the relative strength of the P_{WT} promoters, except *bla_{TEM-1}*, was greater than that of P_{A1-O4/O3}. The *bla_{NDM-5}* promoter was strongest, followed by *bla_{OXA-181}*,

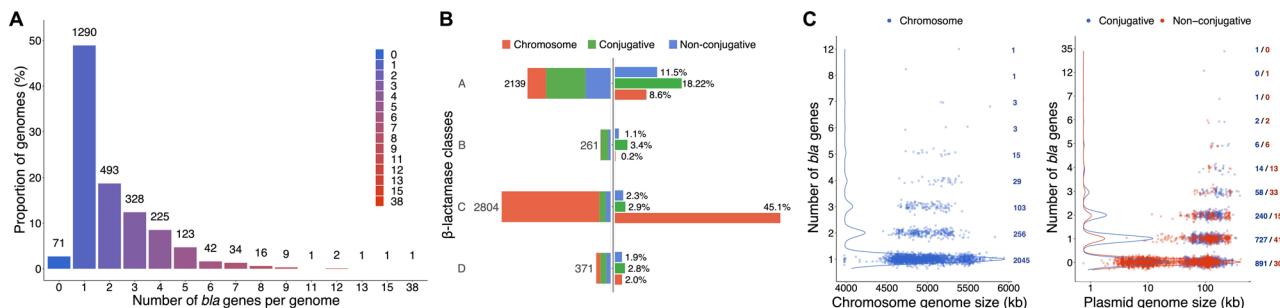


Fig. 1 | The distribution of β -lactamase genes in *Escherichia coli* genomes. **A** A survey of the *bla* gene in *E. coli* genomes ($n = 2637$). Colors ranging from blue to red represent an increasing number of genes co-existing in *E. coli* genomes. **B** Distribution of the four classes (A, B, C, and D) of *bla* genes in *E. coli* genomes. Red, green, and blue represent *bla* genes located on chromosomes, conjugative plasmids, and non-conjugative plasmids, respectively. The numbers indicate the gene count

for each *bla* class, while the percentages represent the gene distribution proportion. **C** A scatterplot of *E. coli* chromosomes or plasmids (conjugative and non-conjugative) according to their genome size and the number of *bla* genes they encode. The density lines depict the distribution of genes per genome. The number of genomes with the corresponding number of genes is shown alongside and colored accordingly.

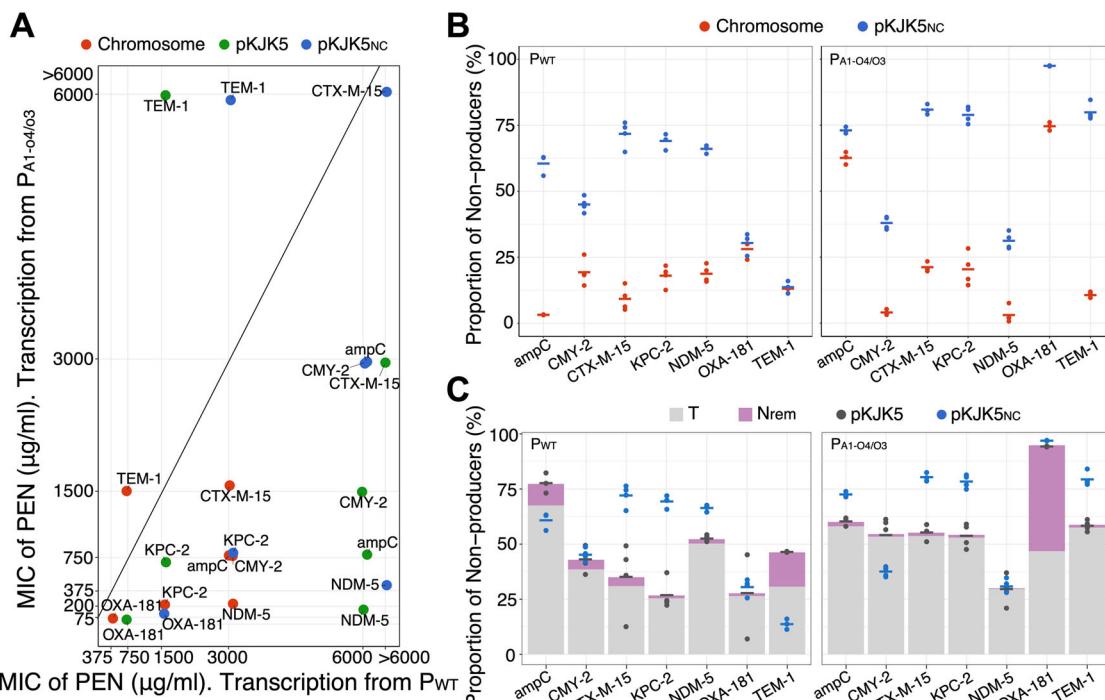


Fig. 2 | Promoter strength comparison and cooperative antibiotic resistance of seven different β-lactamases. A Minimum inhibitory concentrations (MIC, μg/ml) of penicillin (PEN) for *E. coli* encoding different β-lactamases either in the chromosome (red), a conjugative plasmid pKJK5 (green), or a non-conjugative plasmid pKJK5_{NC} (blue). The X-axis is the MIC value of β-lactamase transcribed from the associated wild-type promoters (P_{WT}). The Y-axis is the MIC value of β-lactamase transcribed from a synthetic promoter (P_{A1-O4/O3}). B In the presence of PEN, the proportion of rescued non-producers in coculture with β-lactamase producers (encoded in the chromosome (red), and non-conjugative plasmid pKJK5_{NC} (blue)). The mean values are compared using a *t*-test with false discovery rate controlled.

C In the presence of PEN, the proportion of rescued non-producers in coculture with different β-lactamase producers (encoded in the conjugative plasmid pKJK5 (black), and non-conjugative plasmid pKJK5_{NC} (blue)). The bar chart shows the proportion of rescued non-producers in coculture with the conjugative plasmid producers. The transconjugants (T, gray) and plasmid-free non-producers (Nrem, orchid) are shown. For (B) and (C), the first panels present β-lactamases transcribed from P_{WT}, and the second panels show those transcribed from P_{A1-O4/O3}. Cocultures were initiated with a 1:1 ratio of producer to non-producer. Colored dots represent individual data points ($3 \leq n \leq 5$). Horizontal lines represent mean values.

*bla*_{ampC}, *bla*_{CMY-2}, *bla*_{KPC-2}, and *bla*_{CTX-M-15}. This was consistent for MICs of CEF (Supplementary Fig. 4B). As expected due to higher copy numbers, MIC values of plasmid-carrying producers were higher than those of chromosomal producers, regardless of the promoter (Supplementary Fig. 4C, D).

Currently, little experimental evidence exists on the comparative differences between different types of β-lactamases as to their level of cooperative resistance. Therefore, we tested the level of rescue that the different β-lactamase producers conferred by co-culturing with an otherwise isogenic β-lactam sensitive non-producer (*E. coli* MG1655-*mCherry*⁸), when exposed to PEN or CEF (Fig. 2B and Supplementary Fig. 3B). Overall, the different β-lactamases all rescued the sensitive *E. coli*, demonstrating cooperative resistance, however, to varying degrees (for PEN, Fig. 2B P_{WT} and for CEF, Supplementary Fig. 3B P_{WT}) and the rescue effect of chromosomal producers was generally lower than plasmid-carrying producers ($P_{adj} < 0.05$). Strains with *bla* genes transcribed from P_{A1-O4/O3} (for PEN, Fig. 2B P_{A1-O4/O3} and for CEF, Supplementary Fig. 3B P_{A1-O4/O3}) also varied in their ability to rescue the non-producers, suggesting that some types of β-lactamases provide more cooperative resistance than others in cocultures. When exposed to PEN, *Bla*_{CMY-2} and *Bla*_{NDM-5} producers generally rescued low proportions of non-producers while *Bla*_{ampC} and *Bla*_{OXA-181} producers rescued high proportions, both if encoded in the chromosome or plasmid (Fig. 2B P_{A1-O4/O3}). *Bla*_{CTX-M-15}, *Bla*_{KPC-2}, and *Bla*_{TEM-1} producers facilitated high proportions of non-producers when encoded in the plasmid, while moderate levels when in chromosomes (Fig. 2B P_{A1-O4/O3}). These results support our expectation that a higher copy number of cooperative genes contributes to better cooperative resistance⁸. Differences in cooperative

resistance potentials among the plasmid-carrying producers likely reflect possible variations in the fitness cost of expressing the different β-lactamases and/or variations in affinities toward PEN among the different β-lactamases.

Cooperative resistance promotes conjugative transmission of *bla* genes, withal in the presence of inhibitory concentrations of β-lactams

Conjugation is an important mechanism by which antibiotic resistance is spread horizontally²⁵, yet, in the presence of inhibitory concentrations of antibiotics, transfer is typically hindered as potential recipients succumb before receiving the plasmid. However, this was shown not to be the case for cooperative resistance as rescued recipients efficiently become transconjugants⁸, a phenomenon with potentially critical implications for the spread of resistance genes. Thus, we coculture the non-producer with the different producers carrying the conjugative plasmid encoding the different P_{A1-O4/O3}-*bla* genes, and quantified proportions of non-producers and transconjugants (Fig. 2C and Supplementary Fig. 3C). Overall, the abundances of transconjugants among the original non-producer populations were very large (average 87.9%, Supplementary Table 2) meaning that conjugation efficiently spread the plasmid among the rescued non-producers in the presence of PEN and CEF, for all types of β-lactamases. Comparing the proportions of non-producers rescued (including transconjugants) in cocultures with the conjugating (Fig. 2C, black) and not conjugating (Fig. 2C, blue) plasmids, we generally observed that the proportions of non-producers were lower when the plasmid was conjugative. This suggests that the plasmid generally imposed a burden on the newly transconjugated hosts, thereby reducing their growth.

Extracellular β -lactamase activity is the least strong delineator of cooperative resistance in cocultures

β -lactamases in gram-negative bacteria are typically located in the periplasmic space²⁶. However, it is also well documented that β -lactamases can be present extracellularly, for example within vesicles^{27,28}, and this is potentially an important characteristic associated with their cooperative properties.

Intracellular and extracellular β -lactamase activities were measured for all strains and varied considerably (Supplementary Fig. 5). When including all data a positive correlation was found between intracellular and extracellular β -lactamase activities, suggesting a relationship between the two (Supplementary Fig. 6). Next, MIC values and β -lactamase activities were plotted as functions of proportions of non-producers in cocultures, excluding those with the conjugative plasmid (Fig. 3 and Supplementary Fig. 7). Using a Spearman's Rank Correlation test and looking at individual *bla* genes (Fig. 3A and Supplementary Fig. 7A), we find that the proportions of non-producers were positively correlated both with MIC (PEN [rho = 0.839, P < 0.001] and CEF [rho = 0.758, P < 0.001], using repeated measures correlation), total β -lactamase activities (PEN [rho = 0.923, P < 0.001] and CEF [rho = 0.773, P < 0.001]), and extracellular β -lactamase activities (PEN [rho = 0.774, P < 0.001] and CEF [rho = 0.685, P < 0.001]). For both PEN and CEF experiments, rho-values were the lowest for correlations between proportions of non-producers and extracellular β -lactamase activities. While subtle, this showed that extracellular β -lactamase activity had the least strong monotonic relationship with proportions of non-producers, inferring that extracellular β -lactamase activity was the least important delineator of cooperative β -lactamase resistance in cocultures. To further assess overall tendencies, the respective data from the MIC, total β -lactamase activity, and

extracellular β -lactamase activity experiments were pooled and putative correlations were analyzed (Fig. 3B and Supplementary Fig. 7B). Based on the pooled data, correlations were not statistically significant, neither between proportions of non-producers and MIC (PEN [rho = 0.387, P = 0.158] & CEF [rho = 0.498, P = 0.074]), total β -lactamase activity (PEN [rho = 0.108, P = 0.340] & CEF [rho = 0.121, P = 0.020]), nor extracellular β -lactamase activity (PEN [rho = -0.052, P = 0.621] and CEF [rho = -0.030, P = 0.127]). Even so, the trend for the pooled data was consistent with the non-pooled data, and the lowest rho-values were found for correlations between proportions of non-producers and extracellular β -lactamase activity.

Cross-protection can establish, and extracellular β -lactamase activity facilitates a legacy effect of cooperative resistance

All tested β -lactamases enabled cooperative resistance toward β -lactams PEN and CEF, rescuing non-producers. Still, specific β -lactamases can provide resistance toward distinct types of β -lactams, and may thus enable cross-protection between different producers or potentiate a legacy effect of cooperative resistance. Three strains with *bla* genes encoded in chromosomes, *P_{chr}-bla_{KPC-2}*, *P_{chr}-bla_{NDM-5}*, and *P_{chr}-bla_{CTX-M-15}*, were selected as these provided resistance toward three distinct β -lactam antibiotics from different families; carbapenem, monobactam, and cephalosporin, respectively. MICs of the three strains showed that the cephalosporin cefotaxime (CTX) at 10 μ g/ml strongly suppressed the growth of *P_{chr}-bla_{KPC-2}*, the monobactam aztreonam (AZT) at 1 μ g/ml effectively inhibited the growth of *P_{chr}-bla_{NDM-5}*, and the carbapenem imipenem (IMP) at 0.4 μ g/ml completely inhibited the growth of *P_{chr}-bla_{CTX-M-15}* (Supplementary Fig. 8 and Supplementary Table 3).

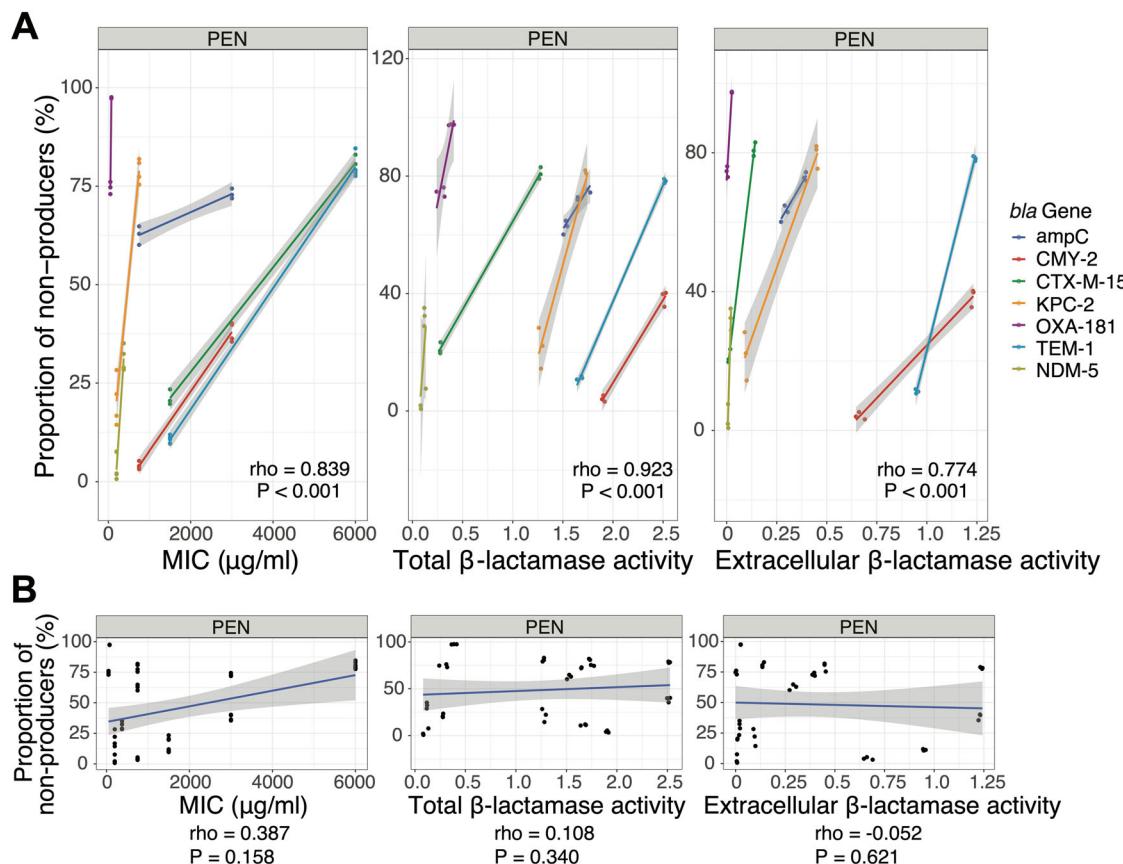


Fig. 3 | Minimal inhibitory concentrations and β -lactamase activities are positively correlated with cooperative resistance. Correlations between the proportion of non-producers and MIC, total β -lactamase activity, and extracellular β -lactamase activity in the presence of PEN. Data is based on strains with *bla* genes in the chromosome (one copy) and the non-conjugative plasmids (multiple copies).

A Correlations stratified by genes. Different colored lines indicate different β -lactamase genes, and dots represent different replicates (3 ≤ n ≤ 5). B Correlations not stratified by gene (all data pooled). Extracellular β -lactamase activity does not correlate positively with the proportions of non-producers.

The three strains were inoculated as mono- and cocultures in a range of concentrations of IMP and AZT or IMP and CTX (Supplementary Fig. 9). Mono-cultures of *P_{chr}-bla_{CTX-M-15}* only grew in a very limited range of concentrations when combined with antibiotics (Fig. 4A and Supplementary Fig. 10A). We note that there was a small variation of IMP MICs for *P_{chr}-bla_{CTX-M-15}* between experiments (Fig. 4A: 0 & 0.1 and Supplementary Fig. 10: 0.2). In contrast to mono-cultures of *P_{chr}-bla_{CTX-M-15}*, growth was more pronounced in cocultures with *P_{chr}-bla_{CTX-M-15}* and *P_{chr}-bla_{KPC-2}* at much larger ranges of concentrations of the two β -lactams (Fig. 4A). This was also the case in cocultures with *P_{chr}-bla_{CTX-M-15}* and *P_{chr}-bla_{NDM-5}* (Supplementary Fig. 10A). Despite the small variation of *P_{chr}-bla_{CTX-M-15}* IMP MICs, the trends were consistent and the results show that complementary cross-protection can establish when strains co-occur. By comparison, mono-cultures of *P_{chr}-bla_{KPC-2}* grew well when exposed to IMP and AZT, but in combinations of IMP and CTX, growth yields were reduced (Fig. 4B, C). Similarly, *P_{chr}-bla_{NDM-5}* grew well in IMP and CTX, while growth was markedly reduced in IMP and AZT (Supplementary Fig. 10B, C). This is consistent with the observation that *P_{chr}-bla_{KPC-2}* is susceptible to CTX in monoculture, as well as *P_{chr}-bla_{NDM-5}* being susceptible to AZT in monoculture (Supplementary Fig. 8).

To further explore the role of extracellular β -lactamase activity on cooperative resistance, we extracted the supernatants from mono-cultures of *P_{chr}-bla_{CTX-M-15}* (*S_{chr}-bla_{CTX-M-15}*), *P_{chr}-bla_{KPC-2}* (*S_{chr}-bla_{KPC-2}*), and *P_{chr}-bla_{NDM-5}* (*S_{chr}-bla_{NDM-5}*) after 24 h of growth (Supplementary Fig. 9B). The filtered *S_{chr}-bla_{KPC-2}* and *S_{chr}-bla_{NDM-5}* were supplemented to the growth medium of mono-cultures of *P_{chr}-bla_{CTX-M-15}* and cultivated in a range of concentrations of IMP and CTX or IMP and AZT (Fig. 4A and Supplementary Fig. 10A). Filtered *S_{chr}-bla_{CTX-M-15}* was supplemented to the growth medium of mono-cultures of *P_{chr}-bla_{KPC-2}* and *P_{chr}-bla_{NDM-5}* and cultivated in a range of concentrations of IMP and CTX or IMP and AZT

(Fig. 4B, C, Supplementary Fig. 10B, C). The supernatants varied markedly in their capacity to rescue the different susceptible bacteria. *S_{chr}-bla_{KPC-2}* enabled the growth of *P_{chr}-bla_{CTX-M-15}* in the presence of IMP ($>3 \mu\text{g/ml}$) and AZT ($>50 \mu\text{g/ml}$), or IMP ($>6 \mu\text{g/ml}$) and CTX ($>100 \mu\text{g/ml}$), concentrations approximately 15-fold higher than the MICs of the *P_{chr}-bla_{CTX-M-15}* strain (Fig. 4A). *S_{chr}-bla_{NDM-5}* facilitated the growth of *P_{chr}-bla_{CTX-M-15}* moderately, that now grew in up to $0.5 \mu\text{g/ml}$ IMP and up to $8 \mu\text{g/ml}$ AZT (Supplementary Fig. 10A). *S_{chr}-bla_{NDM-5}* did not increase the concentration at which *P_{chr}-bla_{CTX-M-15}* could grow in β -lactam combinations with IMP. Curiously, however, the supernatant did increase the range of concentrations of CTX that the strain could grow in when in combination with $0.2 \mu\text{g/ml}$ IMP. The range increased from $20 \mu\text{g/ml}$ CTX to $90 \mu\text{g/ml}$ (Supplementary Fig. 10A). In contrast, due to the sensitivity of *P_{chr}-bla_{CTX-M-15}* to IMP (Supplementary Fig. 8) and its low extracellular β -lactamase activity (Supplementary Fig. 5), supplementing with its supernatant (*S_{chr}-bla_{CTX-M-15}*) did not affect the concentrations of β -lactam combinations, at which neither *P_{chr}-bla_{KPC-2}* nor *P_{chr}-bla_{NDM-5}* could grow (Fig. 4B, C, Supplementary Fig. 10B, C).

The above is consistent with the extracellular β -lactamase activities measured for *P_{chr}-bla_{KPC-2}*, which were significantly higher than both *P_{chr}-bla_{CTX-M-15}* and *P_{chr}-bla_{NDM-5}* ($P < 0.001$, *t*-test). To assess if contamination by the resistant strain or selection of resistant mutants could have caused the observed changes, the MIC of IMP was tested on selected cultures isolated from the supernatant experiment. No changes in MICs were observed, supporting that the changes were facilitated via the supernatants (Supplementary Fig. 11), and implying that the cells that survived under high-concentration dual- β -lactam conditions may have been in a persister-like state. Collectively, the cross-protection experiment suggests that β -lactamases vary markedly in their ability to impact ecosystems by imposing resistance legacy effects, which, for example, could occur during migration-

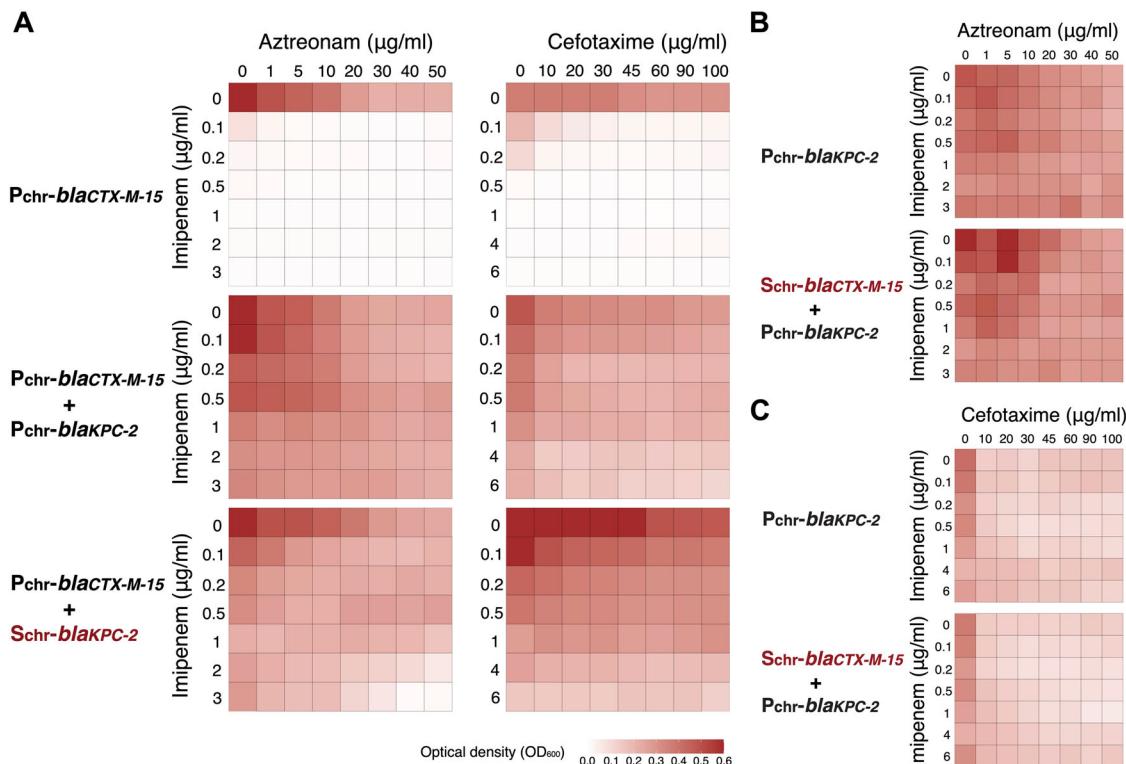


Fig. 4 | Cross-protection can be established in the presence of dual antibiotics, and some β -lactamases protect efficiently extracellularly. **A** Growth yields in different concentrations of dual antibiotics: Aztreonam (AZT) with imipenem (IMP), and Cefotaxime (CTX) with imipenem (IMP). For each combination, three culture conditions were tested: Mono-cultures of *P_{chr}-bla_{CTX-M-15}*, Cocultures of *P_{chr}-bla_{CTX-M-15}* and *P_{chr}-bla_{KPC-2}*, Mono-cultures of *P_{chr}-bla_{CTX-M-15}* supplemented

with *S_{chr}-bla_{KPC-2}*. **B** Growth yields of mono-cultures of *P_{chr}-bla_{KPC-2}* in different concentrations of AZT and IMP. Two culture conditions were tested: No supplementation with *S_{chr}-bla_{CTX-M-15}*, and Cultures supplemented with *S_{chr}-bla_{CTX-M-15}*. **C** Growth yields of mono-cultures of *P_{chr}-bla_{KPC-2}* in different concentrations of CTX and IMP. Two culture conditions were tested: No supplementation with *S_{chr}-bla_{CTX-M-15}*, and Cultures supplemented with *S_{chr}-bla_{CTX-M-15}*.

emigration events. Moreover, a resistance legacy effect also has the potential to corroborate the resistance phenotype when producer population densities fluctuate over time.

Discussion

Here, we explored the diversity and genomic context of *bla* genes bioinformatically among *Escherichia coli* and observed an especially strong association between *bla* genes and conjugative plasmids, and disclosed the abundances of β -lactamase types. Based on this, seven relevant *bla* genes representing the four known β -lactamase classes were focused on to study the cooperative properties of the different β -lactamases. The presented experimental data imply that cooperative resistance is universal among β -lactamases, yet the level at which susceptible cells were rescued varied markedly. We show that elevated expression of any of the β -lactamases increased the cooperative resistance, and this could facilitate cross-resistance between different β -lactamase producers. Our results also showed that both the intracellular and extracellular activities of the different β -lactamases played a defining role in cooperative resistance in cocultures. However, extracellular β -lactamase activity was specifically an important underlier of resistance legacy effects, which we speculate may be important during migration-emigration events and when producer population densities fluctuate over time.

In silico analyses showed that *bla* genes are widespread among *E. coli*, of which the class A β -lactamases are the most common after excluding *bla_{ampC}* and *bla_{ampC1}* genes. Although *bla_{ampC}* and *bla_{ampC1}* genes typically are not expressed in *E. coli*, their high abundance may increase the risk that *bla_{ampC}* spreads horizontally and becomes functional through mutations. In particular, mutations of their promoters can lead to *bla_{ampC}* expression and β -lactam resistance²⁹. The distribution of *bla* genes on chromosomes and plasmids shows a strong association with conjugative plasmids. For example, the carbapenem-resistant genes of classes B and D were abundantly present on conjugative plasmids, which may lead to their rapid spread. Also, although most plasmids only carried a single *bla* gene, some of them could maintain as many as 35 genes at the same time, thus indicating that an association between *bla* genes and plasmid replicons can be highly successful and could be linked to the cooperative properties of the β -lactamases⁸.

Furthermore, we found that the wild-type promoters of the different β -lactamases were generally stronger than the $P_{A1-O4/O3}$ promoter, except for *bla_{TEM-1}*, which had similar strength. The high expression of the β -lactamase genes from strong promoters is consistent with selection toward high levels of resistance³⁰. We find that strong expression of *bla* genes resulted in stronger cooperative resistance, and this in turn promotes conjugative plasmid transfer. The β -lactamases expression from strong promoters may thus be a plasmid self-promoting trait. We have previously found that high cooperative resistance can be a disadvantage when encoded chromosomally in the presence of non-producing cheats⁸.

Although extracellular β -lactamase activity is often considered central to cooperative resistance, our results indicate that total β -lactamase activity, including intracellular contributions, is a stronger determinant in cocultures, with extracellular activity alone having a more limited role. This was based on coculture experiments and highlights that intracellular β -lactamase activity is also important for reducing the concentration of β -lactams outside the cells. In support of this important distinction, cooperative resistance caused by intracellular antibiotic modification, or degradative enzymes, has consistently been reported for chloramphenicol acetyltransferases³¹. Chloramphenicol-resistant bacteria can express chloramphenicol acetyltransferase to acetylate chloramphenicol in the cell. This way, large concentrations of chloramphenicol are inactivated intracellularly. Hence, due to diffusion, intracellular acetyltransferases reduce the extracellular chloramphenicol concentration, which can result in the rescue of neighboring susceptible bacteria^{19,32}. These results highlight the need for a more comprehensive understanding of the relationship between intracellular and extracellular β -lactamase activity and cooperative antibiotic resistance, particularly in the context of infections. Our results imply that the

social inter-cellular phenomenon, that is, cooperative resistance, is not necessarily defined by an extracellular public good protein. The public good that β -lactamases provide is instead the removal of β -lactams locally from the extracellular space. That social interactions are not fundamentally defined by an extracellular public good is important to regard when, for example, defining the potential for social interactions bioinformatically, which otherwise may lead to misestimations.

Among the factors tested here, the multi-copy nature of plasmids was found to be important for promoting cooperative antibiotic resistance. Studies have also reported that plasmids offer benefits beyond mobility; for example, multi-copy plasmids provide bacteria with higher expression levels and mutation rates, promoting long-term co-evolution^{18,33}. Consequently, the host bacterium also acquires higher levels of antibiotic resistance³⁴. While multiple copies and increased transfer of plasmids may impose a fitness burden on bacteria, plasmids can evolve to adjust the copy number, achieving maximum fitness with minimum cost³³.

Here we demonstrate that cross-protection between resistant bacteria can help them evade the effects of multiple different β -lactam antibiotics. In line with this, a mutualistic symbiosis of *E. coli* under combined treatment with ampicillin and chloramphenicol has also been found¹⁹. Other studies have, on the other hand, shown that combinations of different β -lactams may corner the evolution of β -lactam resistance³⁵, but such strategies could be sensitive to cross- and multi-resistance. β -lactams are often co-administered with β -lactamase inhibitors, and such combination therapies could possibly affect cross-protecting β -lactamases. Ideally, β -lactamase inhibitors work by irreversibly binding to and hereby inactivating β -lactamases, so their presence would have direct effects also on the cooperative resistance properties³⁶. However, in the presence of multiple β -lactamases during cross-protection, the affinity of a β -lactamase inhibitor toward a specific β -lactamase could obscure such an effect. Limited research has investigated the role of β -lactamase inhibitors on cooperative β -lactamase resistance.

Moreover, we confirmed that extracellular β -lactamases can exist stably and contribute to cross-protection, which emphasizes the potential importance of β -lactamases as public goods in bacterial survival strategies. Public goods have long been recognized as a crucial aspect of bacterial cooperation, facilitating resource sharing and enhancing overall survival rates³⁷. In the cross-protection experiments, we tested the resistance of the protected cells and found that they were indeed sensitive despite surviving. We suggest that this implies that the surviving *E. coli* cells were in a persister state. Since persister cells can serve as reservoirs for the spread and evolution of antibiotic resistance genes³⁸, our findings encourage further explorations of the potential link between persister cell formation and cooperative resistance. Potentially, cooperative resistance has a role in long-term adaptation toward heightened frequencies of persister cell formation.

In conclusion, the cooperative nature of β -lactamases contributes to the intricate dynamics of β -lactam antibiotic resistance among bacteria and how the widespread β -lactam resistance is. Our results expand the general understanding of β -lactamases and highlight knowledge gaps that need to be addressed to fully understand coevolutionary, transmission, and cooperative aspects of β -lactamases. A deeper understanding of cooperative antibiotic resistance has the potential to facilitate more effective implementation of improved therapies, such as combination therapies, in clinical settings.

Materials and methods

Bacterial strains and plasmids

All *E. coli* strains (Supplementary Table 4) were grown in Luria-Bertani (LB) medium with appropriate antibiotics and incubated overnight at 30 °C or 37 °C as specified. For strains carrying β -lactamase genes regulated by wild-type promoters, refer to the previous study for specific molecular cloning and gene source information²³. For strains carrying the β -lactamase gene regulated by promoter $P_{A1-O4/O3}$, vectors pGGR36-sfGFP- $P_{A1-O4/O3}$ -*bla* were constructed using the NEBuilder HiFi DNA Assembly Cloning Kit (NEB #E5520) following the provided protocol. The Tn7 transposable system was then used to add the target gene to attachment sites Tn7 on

chromosomes and plasmids²³. The primers (TAG Copenhagen A/S) used in this study are in Supplementary Table 5.

Antibiotics

The antibiotics used in this study were prepared as follows: Ampicillin (AMP, Sigma-Aldrich A9518) was dissolved in water with a stock concentration of 10 mg/ml and a working concentration of 100 µg/ml. Imipenem (IMP, Sigma-Aldrich I0160) was dissolved in water, with a stock concentration of 5 mg/ml and a working concentration of 4 µg/ml. Cefotaxime (CTX, Sigma-Aldrich C7039) was also dissolved in water, with a stock concentration of 10 mg/ml and a working concentration of 2 µg/ml. Tetracycline (TET, Sigma-Aldrich T3383) was dissolved in 50% ethanol, with a stock concentration of 10 mg/ml and a working concentration of 15 µg/ml. Kanamycin (KAN, Sigma-Aldrich K1377) was dissolved in water, with a stock concentration of 10 mg/ml and a working concentration of 50 µg/ml. Penicillin (PEN, Sigma-Aldrich 13752) was dissolved in water, with a stock concentration of 100 mg/ml and a working concentration of 50 µg/ml. Cephalothin (CEF, Sigma-Aldrich C4520) was dissolved in water, with a stock concentration of 100 mg/ml and a working concentration of 20 µg/ml. Finally, Aztreonam (AZT, Sigma-Aldrich A6848) was dissolved in dimethylformamide with a stock concentration of 50 mg/ml. These concentrations were used in all relevant experiments to ensure consistency and reproducibility.

Determination of antibiotic resistance gene profile

The complete chromosome and plasmid genomes of *E. coli* were downloaded from GenBank on April 22, 2023. To compare β-lactamase genes between the chromosome and plasmid, only isolates containing both the chromosome and at least one plasmid sequence were included. Based on such requirements, genomes from 2637 submissions were analyzed.

β-lactamase genes were identified in each genome (either chromosome or plasmid) based on the Comprehensive Antibiotic Resistance Database (CARD, 3.2.6), with the CARD's Resistance Gene Identifier (RGI) software (6.0.2)²¹. In total, 156038 perfect and strict hits were identified using CARD RGI. Loose RGI hits of 95% identity or better were not included. "DIAMOND" was used as the alignment tool³⁹. β-lactamase genes were classified into classes A, B, C, and D, based on the CARD database. The mobility of the plasmid was determined with the tool "Plasmad"⁴⁰.

β-lactamase activity assay

Intracellular and extracellular β-lactamase activity was determined based on the change in the color assay of nitrocefin hydrolysis. The centrifuged supernatants of overnight broth cultures were used to measure the extracellular β-lactamase activity, and the lysozyme-treated pellets were used to measure the intracellular activity. 20 µl of the treated samples were mixed with 180 µl PBS containing nitrocefin (final concentration of 100 µg/ml) in a 96-well microplate and placed at room temperature for 30 min. The optical density OD₄₉₀ value was detected by a spectrophotometer and recorded. All measurements are parallel three times.

Determination of minimal inhibitory concentrations (MIC)

In this study, MICs were determined using the broth microdilution method. Overnight broth cultures were first washed twice with PBS and diluted to prepare bacterial suspensions. Serial two-fold dilutions of antibiotics (up to 10 concentrations) were added to each row of a 96-well sterile microtiter plate, using LB broth as the antibiotic diluent. It was ensured that the volume of LB broth containing antibiotics in each well was 90 µl. Sterile control wells containing only broth and growth control wells without antibiotics were included in columns 11 and 12. Finally, 10 µl of each bacterial suspension was added to 90 µl of LB broth containing the different concentrations of antibiotics, resulting in a final inoculum of approximately 5 × 10⁵ CFU/mL. The 96-well sterile microtiter plates were then incubated at 37 °C for 16 h, and bacterial growth was assessed the next day by measuring optical density (OD₆₀₀) using a microplate reader (BioTek ELx808). The MIC values of the

strains were defined based on the lowest antibiotic concentration that inhibited the growth of visible bacteria.

E-test

The E-test was conducted following the instruction manual (Biomerieux). First, test strains were collected from 96 wells and washed 3 times with 1× PBS. Then the strains were diluted to 10⁸ CFU/mL (approximately 0.5 McFarland) in PBS. Used a sterile cotton swab to evenly spread the diluted bacterial solution onto the fresh LB agar plate without antibiotics. Once the bacterial solution dried, the ETEST-imipenem strip was positioned at the center of the plate. Incubation was carried out overnight at 37 °C for 16 h. The following day, the MIC was obtained based on the concentration mark of the inhibition zone.

Coculture experiments

The coculture experiments were performed in LB agar plates. First, overnight cultures of β-lactamase producers and non-producers were washed twice with PBS, and the OD₆₀₀ was adjusted to 0.2. After mixing producers and non-producers in an initial ratio of 1:1, 10 µl of the mixture was inoculated on LB agar plates with or without antibiotics, 3 replicates for each condition. The antibiotics used here are penicillin (50 µg/ml, ~120% MIC) and cephalothin (20 µg/ml, ~120% MIC). After one day of incubation at 37 °C, colonies were collected and counted by flow cytometry. The β-lactamase producers expressed sfGFP, which emitted green fluorescence, while the non-producers expressed mCherry, which emitted red fluorescence. Flow cytometry was used to distinguish and enumerate the co-cultured bacteria based on the different fluorescent markers. As the P_{A1-O4/O3} promoter is regulated by LacI, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to ensure full activation of the promoter P_{A1-O4/O3}.

Flow cytometry

Flow cytometric bacterial cell counts were performed on a BD FACSAria Illu (BD Biosciences) using the same technical setup as our previous study⁸. The detection thresholds for forward scatter (FSC) and side scatter (SSC) were set to 200. Data acquisition and analysis were performed by BD FACSDiva software 6.1.3.

The diluted coculture colonies were run at a flow rate of 1 for 1 min, and the fluorescence signal of the cells was collected to count the different cells in the samples. Green (sfGFP) and red (mCherry) fluorescent bacterial cells were gated on bivariate contour plots using FITC and PE-Texas Red regions, respectively. The sfGFP fluorescence was detected with a 488 nm excitation laser and a FITC detector (530/30 nm band-pass filter), while the mCherry fluorescence was detected with a 561 nm excitation laser and a PE-Texas Red detector (610/20 nm band-pass filter). To set gates for green and red fluorescence, the non-fluorescent wild-type *E. coli* MG1655 strain was used as a negative, alongside mono-cultures of producers (green fluorescence) and non-producers (red fluorescence). In coculture experiments, producers (green fluorescence), non-producers (red fluorescence), and transconjugants (with both green and red fluorescence) were counted according to this gating strategy.

Cross-protection experiments

First, individual colonies of experimental strains were picked from fresh agar plates and cultured overnight in LB broth containing supplemented antibiotics. Then the following steps were performed: (a) 10 µl of the overnight cultures were separately inoculated into 5 ml of antibiotic-free LB broth and incubated for 24 h, 250 rpm at 37 °C. (b) Another set of 10 µl of the overnight cultures was separately inoculated into 5 ml of LB liquid containing an appropriate antibiotic supplement and incubated for 4 h, 250 rpm at 37 °C, ensuring the bacteria were in the logarithmic growth phase for highest fitness.

Centrifuged at 2000 × g for 30 min to obtain the fresh supernatant of the 24-h culture in (a). The supernatant was filtered through a 0.2 µm filter and stored on ice. Centrifuged at 8000 × g for 2 min to obtain the pellet of the

4-h culture in (b). Washed the pellet twice with 1× PBS, discarded the supernatant, and also placed the pellet on ice for later use.

Prepare bacterial suspension: For the single-strain culture, the pellet of a single strain was suspended in fresh LB broth and diluted to 10⁷ CFU/ml cells. For the coculture of two strains, the pellets of the two strains mixed in a 1:1 ratio were suspended in fresh LB broth and diluted to 10⁷ CFU/ml cells. For the strain and supernatant coculture, the bacterial pellet was suspended in fresh filtered 24-h culture supernatant and diluted to 10⁷ CFU/ml cells.

Cross-protection experiments were performed using a 96-well sterile microtiter plate. Specifically, different concentrations of antibiotics were added to each row, and another set of different concentrations of antibiotics was added to each column. Finally, 10 µl of each suspension was added to 90 µl LB broth containing varying concentrations of 2 antibiotics. Incubated at 37 °C for 16 h, and then the growth status of the cultures was measured based on the optical density (OD₆₀₀).

Statistics and reproducibility

The correlation analysis was performed with either the Spearman correlation (R-package “Hmisc”, function “rcorr”), or the repeated measures correlation (R-package “rmcorr”, function “rmcorr”) as specified⁴¹. We used repeated measures correlation to account for the repeated measures of the same genes for their β-lactamase activity, MIC, and proportions of surviving non-producers. The linear regression was obtained with the function “lm” in R-package “stats”. The shaded area of the regression line represents the 95% confidence interval of the mean. The kernel density of β-lactamase gene numbers per genome is estimated and plotted with the function “geom_density” in R-package “ggplot2”. The two-sided Welch’s *t*-test was performed using R-package “stats” with the function “t-test”. The false discovery rate was controlled using the Benjamini-Hochberg method for multiple comparisons, and the adjusted *P*-values were shown as “*P_{adj}*”. Sample sizes and numbers of replicates are provided in each figure legend and represent at least three independent experiments. The source data underlying the main figures are available in Supplementary Data 1–3.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All the data supporting the main text of this study are available in this article and the Supplementary Information files. All other data are available from the corresponding author upon reasonable request.

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Author contributions

J.S.M. and Q.W. designed the study. Q.W. carried out the experiments. S.W. retrieved and analyzed genomic data, as well as performed statistical analysis. J.S.M. and Q.W. contributed to the concept and interpretation of the results. Q.W. drafted the manuscript. J.S.M. and S.W. revised and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Jonas Stenløkke Madsen.

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