



Evolutionary Trajectories toward High-Level β -Lactam/ β -Lactamase Inhibitor Resistance in the Presence of Multiple β -Lactamases

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ABSTRACT β -Lactam antibiotics are the first choice for the treatment of most bacterial infections. However, the increased prevalence of β -lactamases, in particular extended-spectrum β -lactamases, in pathogenic bacteria has severely limited the possibility of using β -lactam treatments. Combining β -lactam antibiotics with β -lactamase inhibitors can restore treatment efficacy by negating the effect of the β -lactamase and has become increasingly important against infections caused by β -lactamase-producing strains. Not surprisingly, bacteria with resistance to even these combinations have been found in patients. Studies on the development of bacterial resistance to β -lactam/ β -lactamase inhibitor combinations have focused mainly on the effects of single, chromosomal or plasmid-borne, β -lactamases. However, clinical isolates often carry more than one β -lactamase in addition to multiple other resistance genes. Here, we investigate how the evolutionary trajectories of the development of resistance to three commonly used β -lactam/ β -lactamase inhibitor combinations, ampicillin-sulbactam, piperacillin-tazobactam, and ceftazidime-avibactam, were affected by the presence of three common β -lactamases, TEM-1, CTX-M-15, and OXA-1. First-step resistance was due mainly to extensive gene amplifications of one or several of the β -lactamase genes where the amplification pattern directly depended on the respective drug combination. Amplifications also served as a stepping-stone for high-level resistance in combination with additional mutations that reduced drug influx or mutations in the β -lactamase gene *bla*_{CTX-M-15}. This illustrates that the evolutionary trajectories of resistance to β -lactam/ β -lactamase inhibitor combinations are strongly influenced by the frequent and transient nature of gene amplifications and how the presence of multiple β -lactamases shapes the evolution to higher-level resistance.

KEYWORDS β -lactam/ β -lactamase inhibitor, antibiotic resistance, evolution, gene amplification

β -Lactams are the most important class of antibiotics used for the treatment of Gram-negative bacterial infections (1). However, their extensive use has led to resistance development, and today, the prevalence of β -lactam resistance among bacterial pathogens has severely reduced their usability for treatment in many parts of the world. Mechanisms of resistance against β -lactams include decreased influx as well as increased efflux of the drug, target alteration, and drug inactivation (2). While drug inactivation by β -lactamase enzymes that hydrolyze the β -lactam ring is the most important clinical resistance mechanism among Gram-negative bacteria, combinations of several resistance mechanisms are common among clinical isolates and contribute to the level of resistance (2). To date, many hundreds of unique β -lactamase genes, either plasmid borne or chromosomally located, have been identified in various contexts (3). Some of the ones most commonly found in clinical isolates belong to the groups TEM, OXA, SHV, and CTX-M (3, 4). Early members of the TEM and OXA enzyme groups mainly catalyze the degradation of penicillins and early-generation cephalosporins,

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The authors declare no conflict of interest.

Received 26 February 2022

Returned for modification 23 March 2022

Accepted 9 May 2022

Published 2 June 2022

but through the accumulation of point mutations, they have developed extended-spectrum activity (named extended-spectrum β -lactamases [ESBLs]) toward later generations of cephalosporins and, in some cases, even against carbapenems (5–7).

With the increased prevalence of ESBL-producing bacteria, combinations of β -lactam antibiotics with β -lactamase inhibitors have become of growing clinical importance (1, 8, 9). The inactivation of the β -lactamase activity prevents the enzyme from acting on the incoming antibiotic, which restores the susceptibility of the cell (10, 11). This approach has attracted much interest from the pharmaceutical industry, and there is an increasing number of new potential β -lactamase inhibitors in the pharmaceutical pipeline (12–15). Three commonly used inhibitors are sulbactam, tazobactam, and avibactam, mainly effective against Ambler class A, C, and D β -lactamases (1).

Following the introduction of β -lactamase inhibitors into clinical practice, resistance against these compounds has been reported (16–21). The main resistance mechanism has been the overexpression of β -lactamases, which enables the bacterium to cope with higher doses of the drugs (16, 17, 19, 20, 22, 23), but single-amino-acid substitutions rendering the β -lactamase less sensitive to the combination treatment have also been found (4, 18, 21, 24). Most studies on inhibitor resistance have demonstrated how individual β -lactamases circumvent the inhibition of combination treatment. However, clinical isolates often encode multiple β -lactamases, often with overlapping catalytic spectra, which may lead to different forms of allogeneous selection (25). It is therefore conceivable that the evolution of resistance to β -lactam/ β -lactamase inhibitor combinations in a strain with several β -lactamases differs from that of a strain carrying only one enzyme variant due to the expanded selective landscape and differences in catalytic activity and inhibitor binding between the enzymes. Hence, here, we explored the evolutionary trajectories toward resistance to different combinations of β -lactam/ β -lactamase inhibitors for a strain carrying a plasmid that encodes three different β -lactamases. The pUUH239.2 plasmid was originally isolated from an ESBL-producing *Klebsiella pneumoniae* clone that caused an outbreak at Uppsala University Hospital in Sweden (26). It carries 13 resistance-associated genes, including three β -lactamases: the two narrow-spectrum β -lactamases TEM-1 (class A) and OXA-1 (class D) and the ESBL CTX-M-15 (class A) (26, 27). The resistance cassette of the plasmid is highly similar to those of plasmids associated with *Escherichia coli* sequence type 131 (ST131), and the plasmid backbone is highly similar to that of the *Klebsiella* plasmid pKPN3 originally isolated from a *K. pneumoniae* ST258 strain (27). To investigate the evolutionary trajectories of resistance to β -lactamase/ β -lactamase inhibitors, we chose three clinically used combinations, (i) ampicillin-sulbactam (SAM), (ii) piperacillin-tazobactam (TZP), and (iii) ceftazidime-avibactam (CZA) (28–30). We conducted one-step selections as well as short-term *in vitro* evolution experiments at increasing concentrations of the antibiotic-inhibitor. Isolated mutants and endpoint populations were characterized by their maximum growth rate, MIC, cross-resistance to other combinations, and whole-genome sequence to elucidate the genetic basis of resistance.

RESULTS

To study the effect of multiple β -lactamases on the development of resistance to β -lactam/ β -lactamase inhibitor combinations, we used the multiresistance plasmid pUUH239.2 (carrying *bla*_{TEM-1}, *bla*_{OXA-1}, and *bla*_{CTX-M-15}) in the well-studied *E. coli* MG1655 strain as a model system (27). The three combinations ampicillin-sulbactam, piperacillin-tazobactam, and ceftazidime-avibactam were chosen due to their use in the treatment of infections caused by *E. coli* (28, 29, 31, 32). Susceptibility to the combinations was determined in two ways throughout the study: (i) according to the standard EUCAST methodology measuring the MIC, where a fixed concentration of 4 mg/L of the inhibitor is used, and (ii) according to the concentration ratio that the drug and the respective inhibitor are administered to treat an infection (called MIC_{ratio} here). The similar pharmacokinetic properties of the paired antibiotic-inhibitor combinations result in a maintained ratio during treatment, but the absolute concentrations will vary depending on the dosing interval and location in the body (33, 34). The MIC_{ratio} therefore relates directly to the selective potential of the clinically used drug ratio at different concentrations, and we therefore used the clinical treatment ratios of the drug and the

TABLE 1 Susceptibility profiles of the parental strains and one-step selection mutants for SAM, TZP, and CZA^a

Strain	Combination for selection	Mean growth rate ± SD	SAM MIC _{ratio} (mg/L)	SAM MIC (mg/L)	TZP MIC _{ratio} (mg/L)	TZP MIC (mg/L)	TZ MIC (mg/L)	CZA MIC _{ratio} (mg/L)	CZA MIC (mg/L)
WT	None	1 ± 0.02	4	4	2	2	0.5	0.25	0.25
WT/pUUH	None	0.97 ± 0.01	32	>256	16	8	64	0.25	0.25
DA61263	SAM	0.84 ± 0.05	256	>256	256	256	0.5	0.25	0.25
DA61264		0.75 ± 0.01	256	>256	256	256	0.5	0.25	0.25
DA61265		0.81 ± 0.01	256	>256	64	256	64	1	0.5
DA61266		0.87 ± 0.02	128	>256	32	128	0.5	0.5	0.25
DA61267		0.99 ± 0.04	256	>256	64	256	64	0.5	0.25
DA61268	TZP	1.00 ± 0.02	64	>256	128	>256	64	1	0.25
DA61269		0.98 ± 0.02	64	>256	64	256	64	1	0.25
DA61270		1.05 ± 0.02	64	>256	128	256	64	1	0.5
DA61271		0.97 ± 0.03	64	>256	128	256	64	0.5	0.25
DA61272		0.97 ± 0.04	64	>256	128	256	128	1	0.25
DA61259	CZA	0.85 ± 0.04	128	>256	64	32	128	2	0.25
DA61260		0.83 ± 0.05	64	>256	32	128	64	4	0.25
DA61261		0.91 ± 0.04	32	128	8	4	8	4	0.5
DA61262		0.56 ± 0.16	128	>256	64	128	>256	2	0.25
DA62411		0.73 ± 0.01	128	>256	64	>256	>256	4	0.25

^aMICs for ampicillin and piperacillin were >256 mg/L for the parental strain and all mutants. Values in boldface type exceed the clinical breakpoints set by EUCAST. Clinical breakpoints are as follows: >8 mg/L for ampicillin-sulbactam (SAM), >8 mg/L for piperacillin-tazobactam (TZP), >4 mg/L for ceftazidime (TZ), and >8 mg/L for ceftazidime-avibactam (CZA). WT, wild type.

inhibitor for the selection of resistant strains. The only combination where there was a significant discrepancy between the MIC and the MIC_{ratio} was SAM, where the plasmid-containing strain had an MIC of >256 mg/L, while the MIC_{ratio} was only 32 mg/L (Table 1). This is explained by the inability of sulbactam to completely inactivate the β-lactamases at the fixed concentration of 4 mg/L used for standard susceptibility testing (Fig. 1A).

Effect of inhibition of β-lactamases. To investigate the relationship between inhibitor concentrations and antibiotic efficacy in response to the three β-lactamases, we expanded the standard susceptibility test by performing a checkerboard analysis with a wider range of inhibitor concentrations. As shown in Fig. 1, the susceptibility for the plasmid-free strain (DA5438) (filled symbols) was largely unchanged for all three antibiotics at the range of inhibitor concentrations tested, apart from at the highest concentrations of sulbactam and avibactam, in accordance with previous reports of the killing effects of these inhibitors at high concentrations (35, 36). For the plasmid-containing strain (DA63522) (open symbols), there was a clear dose dependence of the inhibitor on susceptibility to the antibiotic. Complete inhibition of the β-lactamases (susceptibility equal to that of the plasmid-free strain) required concentrations of 32 mg/L of sulbactam, 64 mg/L of tazobactam, and 1 mg/L of avibactam.

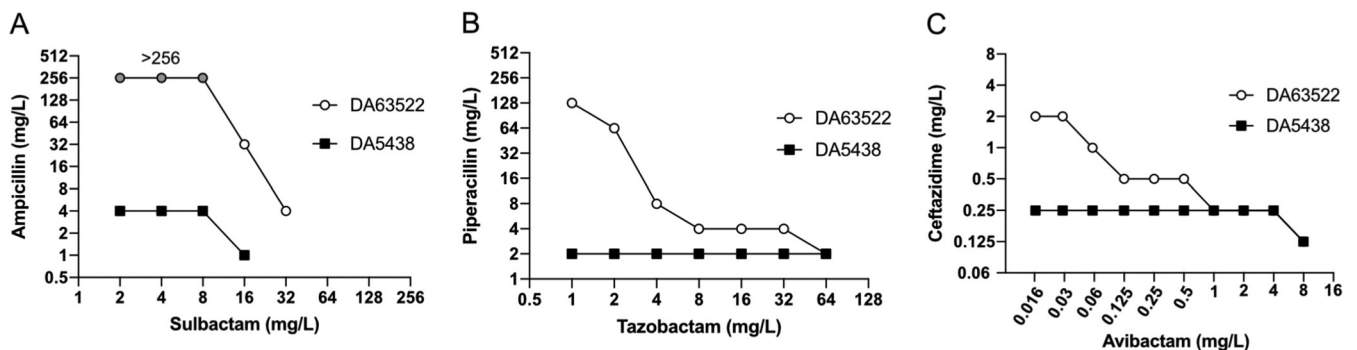


FIG 1 Relationship between β-lactam MICs and inhibitor concentrations. (A) Ampicillin-sulbactam; (B) piperacillin-tazobactam; (C) ceftazidime-avibactam. Filled boxes represent *E. coli* MG1655 (DA5438), and clear circles represent *E. coli* MG1655 containing the pUUH239.2 plasmid (DA63522). Note that for SAM, the ampicillin MIC was >256 mg/L at sulbactam concentrations of <16 mg/L (gray circles), and no growth was detected at sulbactam concentrations of >16 mg/L for DA5438 and >32 mg/L for DA63522. Values are medians from 3 to 4 biological replicates.

There were also clear plateaus in the susceptibility curves, indicating that there is likely a different dose response for the different β -lactamases. Inversely, the highest tested concentrations of inhibitors that gave no detectable effect on the β -lactamases (MIC the same as that without the inhibitor) were 8 mg/L for sulbactam and <1 mg/L for tazobactam, while we never reached a value close to the MIC of ceftazidime (32 mg/L), even with the lowest tested concentration of avibactam (0.016 mg/L). This clearly illustrates the high potency of avibactam to inhibit all three tested β -lactamases even at very low concentrations.

First-step resistance selection. To compare the frequencies and resistance mechanisms for the different drug combinations, we first performed one-step mutant selection on plates containing 1, 2, and 4 times the respective MIC_{ratio5} (Table 1). Mutants were obtained for all of the combinations on the plates at the respective MIC_{ratio} but not above that. The mutant frequencies for SAM and TZP were 4×10^{-9} and 1.7×10^{-9} per cell/division, respectively, whereas for CZA, the frequency was 2×10^{-10} per cell/division. Five clones from each selection were subjected to susceptibility testing, growth rate determination, and whole-genome resequencing to determine genetic changes.

Interestingly, although no mutants could be obtained at concentrations above the MIC_{ratio} , all isolated mutants had increased their MIC_{ratio} 4- to 16-fold, indicating a discrepancy between the resistance levels of the mutants and the mutant-selecting concentrations (Table 1). The MICs for TZP-selected mutants reached the maximum tested concentrations of 256 mg/L, equaling a 32-fold increase. The CZA-selected mutants had MICs that did not differ from those of the parental strain, although the MIC_{ratio} increased 8- to 16-fold. This can be explained by the inhibitor concentration still being much lower than 4 mg/L at these concentrations. Although the parental strain with pUUH239.2 already had a SAM MIC of >256 mg/L, the MIC_{ratio} increased 4- to 8-fold. There was also strong cross-resistance for SAM-selected mutants against TZP (up to a 16-fold increase in the MIC_{ratio}) but less so for TZP-selected mutants against SAM (2-fold increase in the MIC_{ratio}). Some SAM- and TZP-selected mutants had small increases in the MIC or MIC_{ratio} for CZA (2- to 4-fold). Most CZA-selected mutants had increased MICs against SAM and TZP but with substantial variation between mutants. Interestingly, for some mutants, collateral sensitivity was observed, as reported in previous studies (37). For example, three SAM-selected mutants lost their resistance to ceftazidime, and one CZA-selected mutant (DA61261), although with an 8-fold increased MIC_{ratio} for CZA, had an increased susceptibility to ceftazidime alone and generally low MICs for the other combinations. Some selected mutants showed growth defects of up to 40%, but several mutants had no discernible reduction in growth, especially the mutants selected on TZP (Table 1).

Extensive gene amplification of β -lactamase genes causes high-level resistance. We sequenced the first-step mutants to determine the genetic basis for the decreased susceptibility. In accordance with previous studies on resistance to β -lactam/ β -lactamase inhibitor combinations, the majority of isolated mutants had extensive gene amplifications of the plasmid-borne β -lactamase genes that likely result in the overexpression of β -lactamase enzymes (Fig. 2; see also Table S3 in the supplemental material) (20). The three β -lactamase genes are located within a 41-kb resistance region of pUUH239.2 together with other resistance genes and six interspersed copies of IS26 (Fig. 2). Selection for each drug combination resulted in a specific pattern of high-level gene amplifications of the different β -lactamases. SAM mutants had highly increased copy numbers of bla_{TEM-1} (28 to 71 copies), most with amplification recombination points in original or newly inserted IS26 elements (Fig. 2). In addition, three of the SAM mutants had deletions of the regions containing the other two β -lactamases, $bla_{CTX-M-15}$ and bla_{OXA-1} . The mutants selected on TZP showed a different amplification pattern, with a >30-fold gene copy number of bla_{OXA-1} in three mutants. Two mutants, DA61268 and DA61270, instead had complex rearrangements with plasmid sequences inserted on the chromosome in individual positions in rRNA genes through transposition via IS26 elements. DA61268 had a deletion of the *repAB* and *repA4* genes, involved in plasmid replication control, and a deletion of the macrolide resistance region, but apart from that, the whole plasmid appears to have been incorporated on the chromosome. DA61270 had the region between IS26 (2) - IS26 (5), containing both $bla_{CTX-M-15}$ and bla_{OXA-1} .

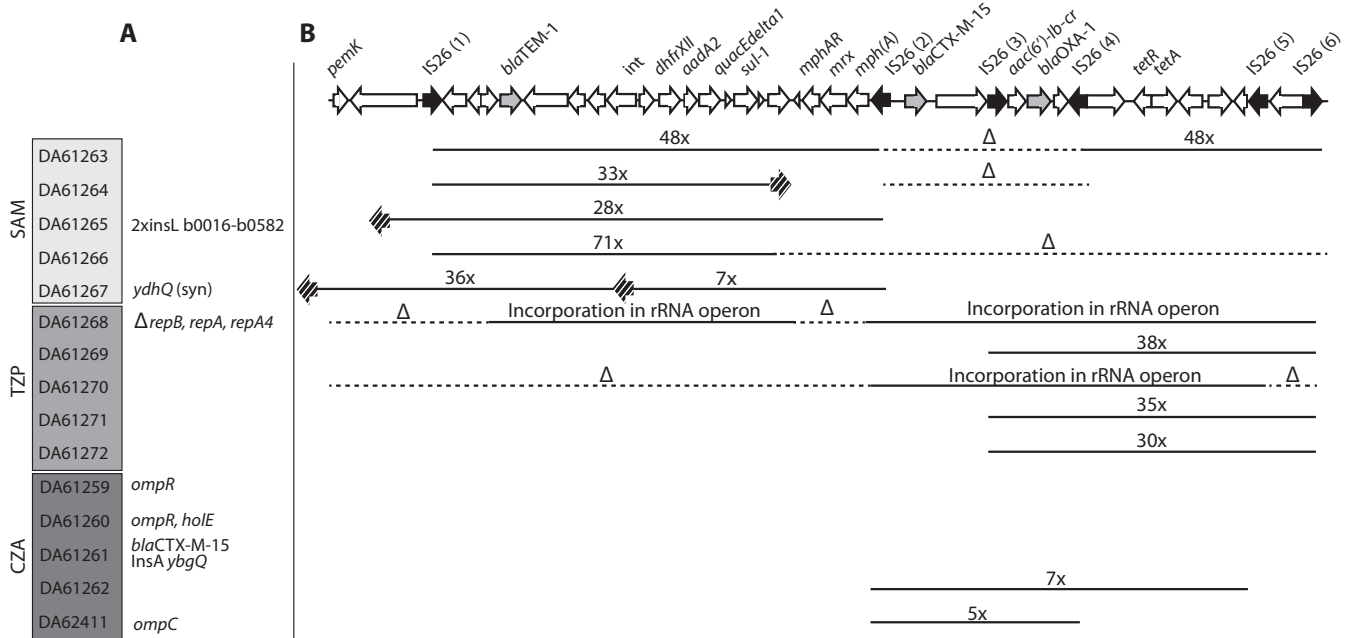


FIG 2 Genetic changes for one-step mutants. (A) Point mutations and other changes. (B) Deletions and amplifications in the resistance cassette. Black arrows indicate IS26 elements, and gray arrows display the three β -lactamases. Striped arrows indicate additional IS26 insertions. Solid lines display the amplification of a certain region of the cassette, whereas dotted lines with Δ indicate deletions. Numbers indicate the copy numbers of the amplified regions. The exact locations of all genetic changes can be found in Table S3 in the supplemental material.

₁₇, inserted into an rRNA operon on the chromosome, while the rest of the plasmid sequences were lost.

The CZA-selected mutants showed a mix of β -lactamase gene amplifications and other mutations. Two mutants had increased copy numbers of *bla*_{CTX-M-15} and *bla*_{OXA-1} (5- and 7-fold) (Fig. 2). In addition, three of the CZA mutants contained mutations in the outer membrane porin OmpC or its regulator OmpR, which has previously been linked to decreased susceptibility to β -lactams (38). Interestingly, DA61261, which showed increased susceptibility to several β -lactams apart from CZA, carried a single-amino-acid change in *bla*_{CTX-M-15} (G238D, according to the Ambler classification [39]).

Individual overexpression of the three β -lactamases results in increased MIC_{ratio} against different drug combinations. To verify that the identified gene amplifications result in increased gene expression of the β -lactamases, we measured the relative transcription levels of a set of the isolated mutants (Fig. 3). DA61263 had a 48-fold gene copy number of *bla*_{TEM-17}, which was mirrored by the 50-fold increased transcription of *bla*_{TEM-1}. DA61269 had a 38-fold copy number of *bla*_{OXA-1} and a corresponding transcriptional increase of more than 20-fold. The two mutants that had inserted plasmid sequences in rRNA operons (DA61268 and DA61270) also showed increased expression of β -lactamases (up to 20-fold), especially *bla*_{OXA-1} (Fig. 3).

Since there appears to be a pattern where increased expression was seen for *bla*_{TEM-1} in SAM-selected mutants, *bla*_{OXA-1} for TZP-selected mutants, and *bla*_{CTX-M-15} for CZA-selected mutants, we analyzed the individual effects of the three β -lactamases using the L-arabinose-inducible pBAD18 expression system. As expected, in the absence of the inhibitor, the overexpression of all three β -lactamases gave very high increases in the MICs for ampicillin and piperacillin and, in the case of CTX-M-15, also for ceftazidime (Table 2). When overexpressed, all three β -lactamases also gave high MIC and MIC_{ratio} values for SAM, but only TEM-1 and OXA-1 were able to achieve MICs equivalent to clinical resistance to TZP (8 mg/L of the antibiotic with 4 mg/L of the inhibitor), in line with the good impact that tazobactam is known to have on CTX-M enzymes (40). However, none of the β -lactamases were able to achieve high-level resistance to the CZA combination when overexpressed, although a decrease in susceptibility could be observed with the expression of TEM-1 and CTX-M-15. These patterns

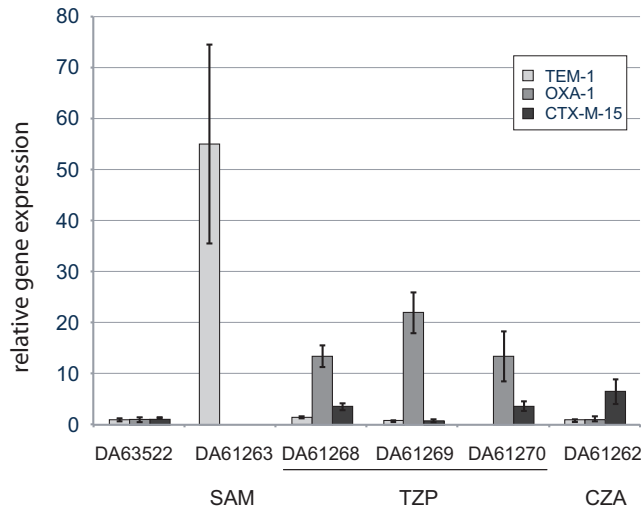


FIG 3 Expression of β -lactamases determined using RT-qPCR in order to verify increased transcript levels. The gene expression of the mutants is relative to that of the parental strain DA63522. Standard deviations from three biological replicates are shown.

are coherent with the pattern of amplifications from the mutants selected on the different drug combinations.

Short-term evolution enables clinical resistance levels for all β -lactam/ β -lactamase inhibitor combinations. While single-step selection on plates allows for rapid identification of initial genetic changes causing decreased susceptibility, it has the limitation that resistance mechanisms requiring combinations of multiple mutations cannot be selected easily. To investigate resistance development in a competitive environment that also allows the selection of multiple mutations, we conducted short-term experimental-evolution experiments in liquid cultures of *E. coli* MG1655 with pUJH239.2 at increasing concentrations of each of the three inhibitor combinations. For SAM and TZP, all lineages were able to achieve high-level resistance, already reaching our upper MIC_{ratio} limit of 256 mg/L in 5 to 8 and 7 to 11 passages, respectively (Fig. 4A and B). The populations passaged in CZA reached a maximum MIC_{ratio} of 8 to 64 mg/L of the antibiotic but reached the clinical resistance breakpoint within 8 to 11 passages (Fig. 4C). All SAM- and TZP-selected lineages and 6/8 CZA-selected lineages had MICs of 256 mg/L, and there was strong cross-resistance to SAM and TZP for all selected lineages (Table 3).

The endpoint populations from all evolved lineages were whole-genome sequenced, and for a subset, we also sequenced populations at earlier time points, when there were shifts in susceptibility, to determine the order in which mutations occurred. The absolute majority of the identified mutations were either nonsynonymous, disruptive (insertions/deletion), or predicted to affect promoters, indicating strong positive selection (Table S3). Due to the strong antibiotic selection pressure, most mutations are likely linked to resistance, but some mutations were likely due to medium adaptations, such as mutations in *malT*, as reported previously (41).

TABLE 2 Effects of the overexpression of the three β -lactamases TEM-1, OXA-1, and CTX-M-15 on different β -lactam/ β -lactamase inhibitor combinations^a

Plasmid	AMP MIC (mg/L)	SAM MIC _{ratio} (mg/L)	SAM MIC (mg/L)	PIP MIC (mg/L)	TZP MIC _{ratio} (mg/L)	TZP MIC (mg/L)	TZ MIC (mg/L)	CZA MIC _{ratio} (mg/L)	CZA MIC (mg/L)
Empty pBAD18	4	4	2	2	2	2	0.25	0.25	0.125
pBAD- <i>bla</i> _{TEM-1}	>256	256	>256	>256	64	8	2	1	0.25
pBAD- <i>bla</i> _{CTX-M-15}	>256	32	64	>256	16	4	256	0.5	0.25
pBAD- <i>bla</i> _{OXA-1}	>256	64	128	>256	128	32	0.25	0.25	0.125

^aAMP, ampicillin; SAM, ampicillin-sulbactam; PIP, piperacillin; TZP, piperacillin-tazobactam; TZ, ceftazidime; CZA, ceftazidime-avibactam. Values in boldface type exceed the clinical breakpoints set by EUCAST.

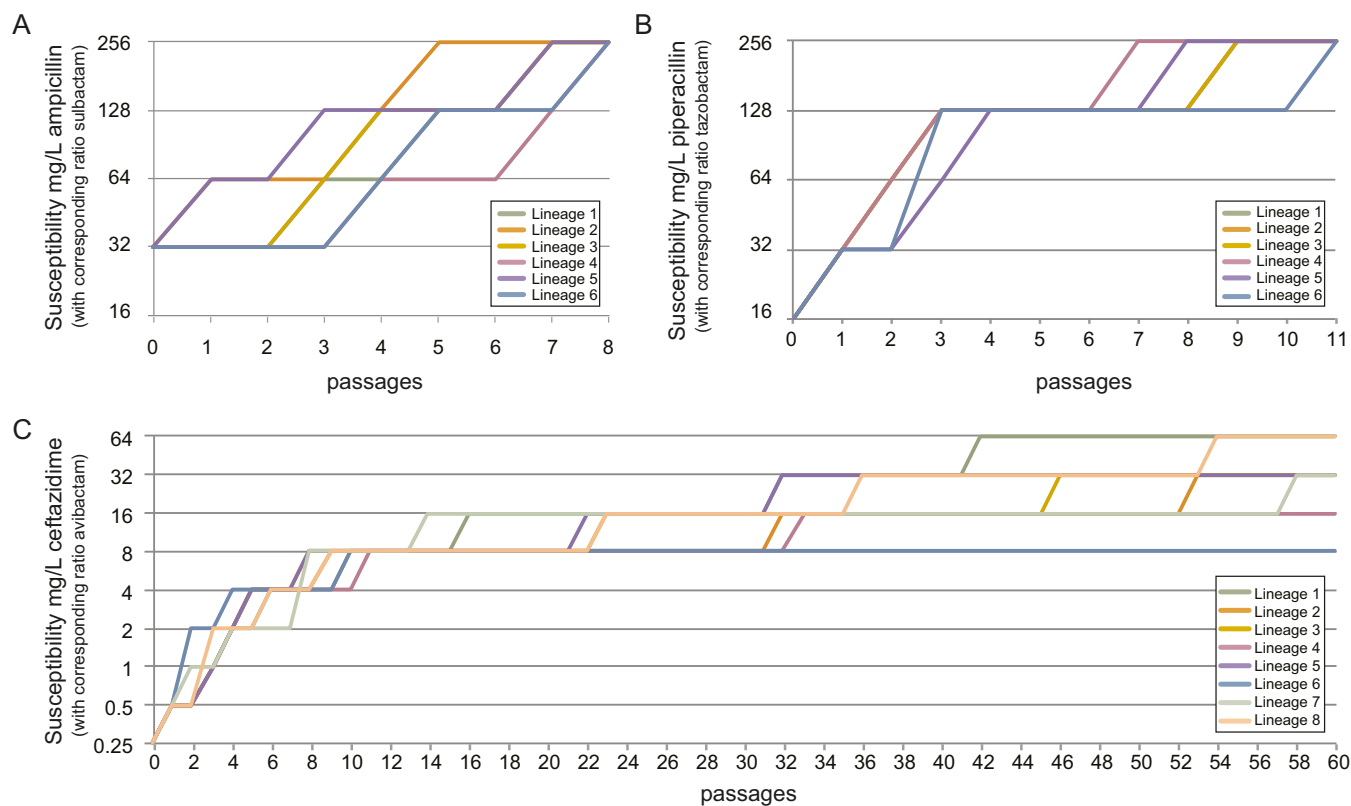


FIG 4 Continuous *in vitro* evolution at increasing concentrations of the three β -lactam/ β -lactamase inhibitor combinations. Curves show the decreases in the susceptibility (MIC_{ratio}) of a strain carrying pUUH239.2 to SAM (A), TZP (B), and CZA (C).

For the populations that evolved in the presence of SAM, the main driver of resistance was again β -lactamase gene amplifications. Especially $bla_{CTX-M-15}$ and bla_{OXA-1} were amplified, which is in contrast to the one-step selection where exclusively bla_{TEM-1} was amplified and where the other two β -lactamases were lost from the plasmid in several cases. We also found subsequent mutations in *ompR*, *ompC*, and lipopolysaccharide (LPS)-associated genes (*rfaC*, *rfaG*, *rclR*, and *galU*) (Fig. 5A and Table S3). The TZP-evolved populations also showed extensive amplifications (up to 52-fold) but involving only bla_{OXA-1} . These amplifications were found already in the first cycle, and the copy number increased further during passaging. For TZP, common mutational targets were also genes involved in the respiratory chain, especially *cydA* and *cydB*, as well as LPS-associated genes (*rfaE*, *rfaG*, and *gmhB*) (Fig. 5B and Table S3). In addition, two of the mutants displayed duplications of different regions on the chromosome spanning approximately 230 and 600 kb, respectively. Both regions have different genes important for cell envelope integrity, such as penicillin binding proteins (PBPs), and the stress response (for exact positions of the duplications, see Table S3).

Evolution in the presence of CZA yielded a more complex picture, where high-level resistance depended on the occurrence of multiple mutations (on average, 15 changes per lineage in the end population, including mutations, deletions, and amplifications) (Fig. 5C and Table S3). This is in accordance with the observation that single-step mutants did not yield high-level CZA resistance. The CZA populations showed early amplifications of $bla_{CTX-M-15}$ and bla_{OXA-1} and mutations in outer membrane porins (*ompF* and *ompC*) and/or their regulators (*envZ* and *ompR*). In five of the lineages, the essential gene *bamA* and/or the nonessential complex partner *bamC*, responsible for the assembly of outer membrane proteins, acquired mutations, one of which leads to a truncation of the protein. Four of the CZA lineages showed chromosomal amplification of regions similar to those of the TZP lineages. In three lineages, there were also mutations in the penicillin binding protein PBP3 (*ftsI*), PBP2 (*mrdA*), or PBP1a (*mrcA*) at later stages of evolution. Interestingly, as in the one-step selections, mutations in CTX-M-15 arose during evolution in three out of eight lineages.

TABLE 3 Susceptibility and growth rates of endpoint clones from evolved lineages^a

Strain	Combination used for selection	Evolution endpoint concn (β-lactam/inhibitor) (mg/L)	Mean growth rate ± SD	MIC (mg/L)		
				SAM	TZP	CZA
DA65027	SAM	256/128	0.46 ± 0.03	> 256	> 256	1
DA65028		256/128	0.52 ± 0.01	> 256	> 256	1
DA65029		256/128	0.50 ± 0.01	> 256	> 256	2
DA65030		256/128	0.52 ± 0.04	> 256	> 256	2
DA65031		256/128	0.62 ± 0.02	> 256	> 256	1
DA65032		256/128	0.55 ± 0.01	> 256	> 256	2
DA65043	TZP	256/32	0.59 ± 0.02	> 256	> 256	0.25
DA65044		256/32	0.40 ± 0.01	> 256	> 256	0.5
DA65045		256/32	0.40 ± 0.01	> 256	> 256	0.25
DA65046		256/32	0.60 ± 0.04	> 256	> 256	0.5
DA65047		256/32	0.47 ± 0.05	> 256	> 256	0.25
DA65048		256/32	0.44 ± 0.01	> 256	> 256	4
DA65159	CZA	64/16	0.32 ± 0.01	> 256	256	256
DA65160		32/8	0.36 ± 0.01	> 256	> 256	256
DA65161		32/8	0.40 ± 0.02	> 256	256	> 256
DA65162		16/4	0.41 ± 0.01	> 256	> 256	32
DA65163		32/8	0.26 ± 0.03	> 256	> 256	> 256
DA65164		8/2	0.66 ± 0.01	> 256	64	16
DA65165		32/8	0.41 ± 0.01	> 256	> 256	> 256
DA65166		64/16	0.31 ± 0.01	> 256	256	> 256

^aSAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CZA, ceftazidime-avibactam. Values in boldface type exceed the clinical breakpoints set by EUCAST. For all combinations, resistance (R) breakpoints were >8 mg/L of the antibiotic with 4 mg/L of the inhibitor.

In two of these lineages, the amino acid change P167T occurred early (Table S3). This particular substitution has previously been observed to give enhanced ceftazidime hydrolysis (42). A second amino acid substitution, N132K, a conserved residue for group A β-lactamases (43), occurred in lineage 8 at the last cycle, together with P167T. In one of the lineages, the same mutation arose as that in the one-step selection mutant G238D, this time together with I142V. This displays the potential of altered CTX-M-15 in the evolution toward decreased susceptibility to CZA. The general evolutionary trajectories for CZA resistance seem to first be either amplification of *bla*_{CTX-M-15} and *bla*_{OXA-1} together with reduced porin expression or mutation of *bla*_{CTX-M-15} followed by amplification of the new allele (Fig. 5C). Subsequently, lineages with amplifications/porin loss accumulated a wide variety of different additional mutations, while the amplified CTX-M-15 mutants all yielded mutations in penicillin binding proteins (Fig. 6).

DISCUSSION

Combination therapy with β-lactam antibiotics and β-lactamase inhibitors has the potential to bring antibiotics back to life where resistance had previously limited the treatment options (44–46). In particular, the newer combination of ceftazidime and the non-β-lactam β-lactamase inhibitor avibactam has attracted great clinical interest. The difference between avibactam and other inhibitors, such as tazobactam and sulbactam, which are so-called suicide inhibitors, is its reversible binding to the β-lactamase (47). The use of these three combinations differs globally due to differences in resistance prevalence, but SAM may be given when traditional β-lactam antibiotics are ineffective but the infecting strain expresses non-extended-spectrum β-lactamases. TZP is instead used for infections caused by ESBL-encoding strains, and CZA is used in treatment regimens where ESBL- or carbapenemase-producing strains are causing the disease (30). However, it is not uncommon that clinical isolates encode multiple different β-lactamases and thereby have a broad spectrum of resistance to this important group of antibiotics. While the effect of, and development of resistance to, β-lactam/β-lactamase inhibitor combinations has been studied for individual β-lactamases, little is known about the effect of multiple enzymes in the same cell. Resistance to SAM has been investigated extensively, where the hyperproduction of, or point mutations in, TEM-1 has been shown to lead to high-level

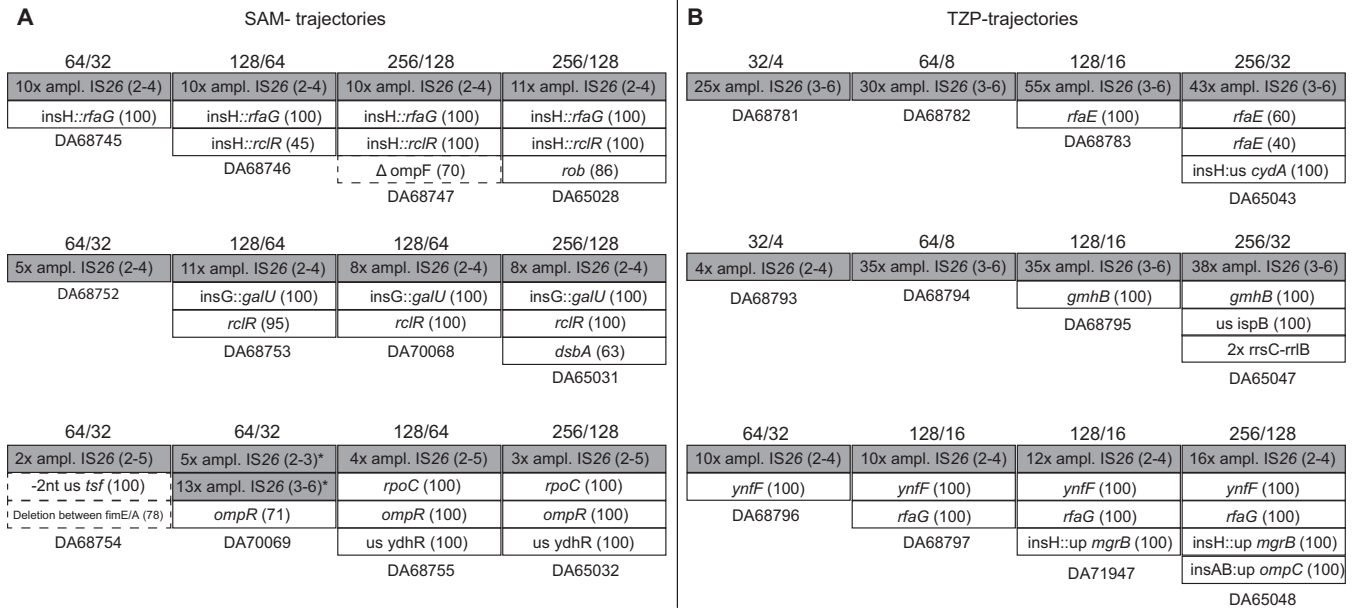


FIG 5 (Continued)

resistance (22, 48, 49). Even though the strain carrying pUUh239.2 is clinically resistant to SAM, by one-step selection, we could see that the amplification of the *bla*_{TEM-1} gene increased the resistance level further and at the same time gave cross-resistance to TZP. However, at the end of the short-term evolution experiments with SAM, the populations showed amplifications of *bla*_{CTX-M-15} and *bla*_{OXA-1}, rather than the *bla*_{TEM-1} gene, indicating that this path is more successful for the bacterium when competing with other possible mutants. The mutation frequencies *in vitro* toward all combinations were relatively low, in the range of 10⁻¹⁰ to 10⁻⁹, indicative of a rare event or combinations of events. This is surprising given that gene duplications, especially between repetitive sequences such as insertion sequence (IS) elements, are several orders of magnitude more frequent than that (50). The likely explanation is that a duplication of a β-lactamase gene helps the cell to survive initially but is not enough to sustain growth at increased concentrations of the antibiotic-inhibitor unless the cell also manages to further increase the copy number significantly, which is a much rarer event. Gene amplification levels of up to 70-fold are very unlikely to spontaneously exist in the population and instead are most likely the result of additional amplification from initial duplications during the growth of cells on the selection plates. This would also explain why we were not able to select mutants at concentrations higher than 1-fold the MIC_{ratio} even though the isolated mutants tolerated much higher concentrations. This means that increased expression of β-lactamases is most likely to evolve at low levels of the drug but can substantially decrease the susceptibility once they have occurred. Previous studies have found the mechanisms of resistance to TZP to be similar to the ones for SAM, where amplifications or promoter mutations of *bla*_{TEM-1} or mutations in CTX-M enzymes increase resistance (17, 20, 37). We found strong cross-resistance to TZP by SAM-selected amplifications of *bla*_{TEM-1} but no such amplifications during TZP selections. Instead, clinical resistance was reached mainly via amplifications of *bla*_{OXA-1} both for the one-step mutants and for populations in the evolution experiments. These mutants also gave cross-resistance to SAM but not CZA. Resistance against CZA previously observed in clinical isolates has been due mainly to point mutations in the carbapenemase genes *bla*_{KPC-2} and *bla*_{OXA-48} (18, 21, 51), while mutations in *bla*_{CTX-M-14} and *bla*_{CTX-M-15} have been found to decrease susceptibility but not enough to reach the clinical breakpoint (18, 52). Here, we found two separate but complementary evolutionary pathways to CZA resistance. Initial changes were either a combination of *bla*_{CTX-M-15} and *bla*_{OXA-1} gene amplifications with loss-of-function mutations in porins or their regulators or initial mutation of *bla*_{CTX-M-15} followed by amplification of the new allele, reduced expression of porins, and mutations in penicillin binding proteins. In addition,

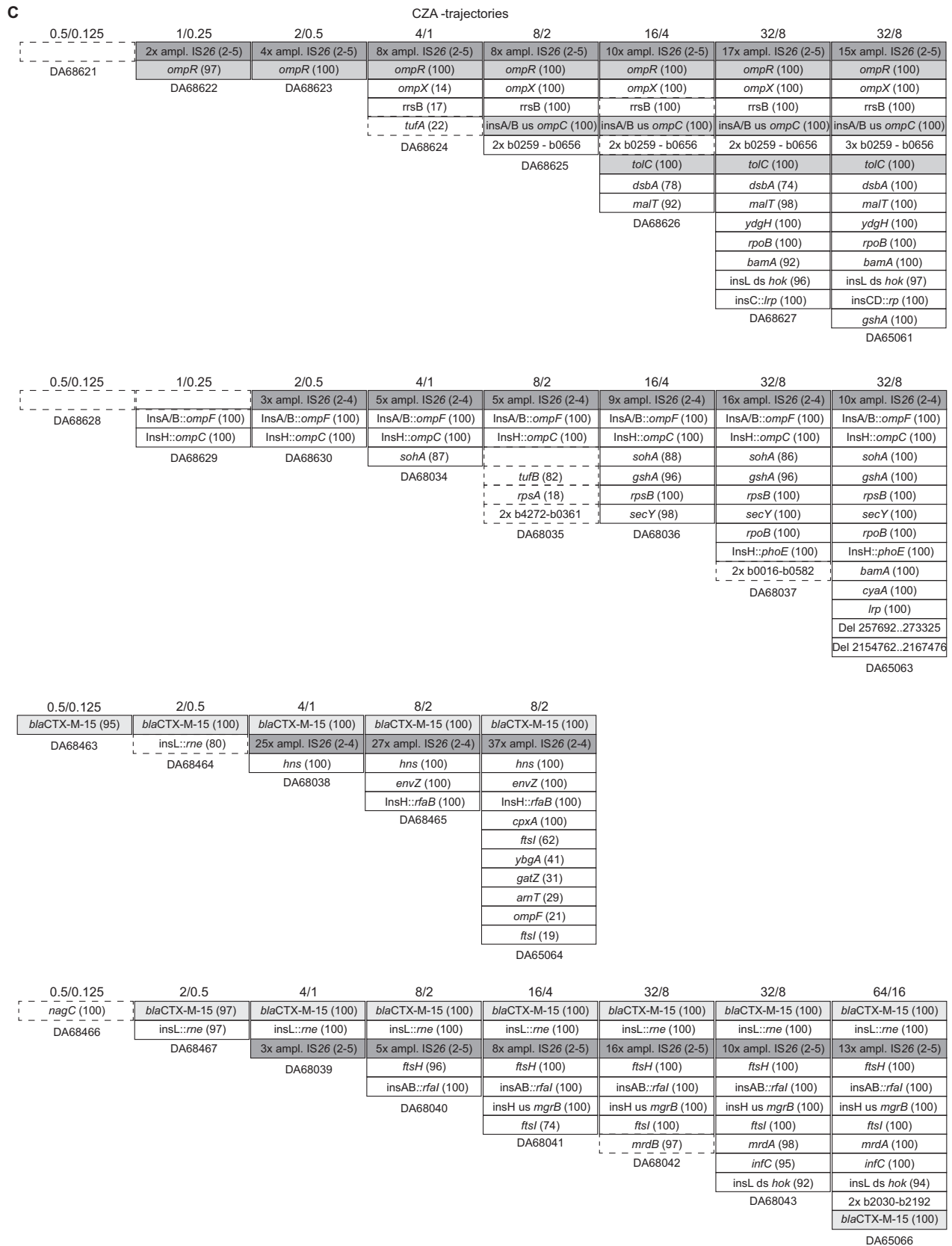


FIG 5 (Continued) Trajectories of the development of resistance to ampicillin-sulbactam (A), piperacillin-tazobactam (B), and ceftazidime-avibactam (C). The accumulation of mutations over time is plotted from left to right. Numbers above the boxes indicate the concentrations of the antibiotic-inhibitor at which the respective population was selected.

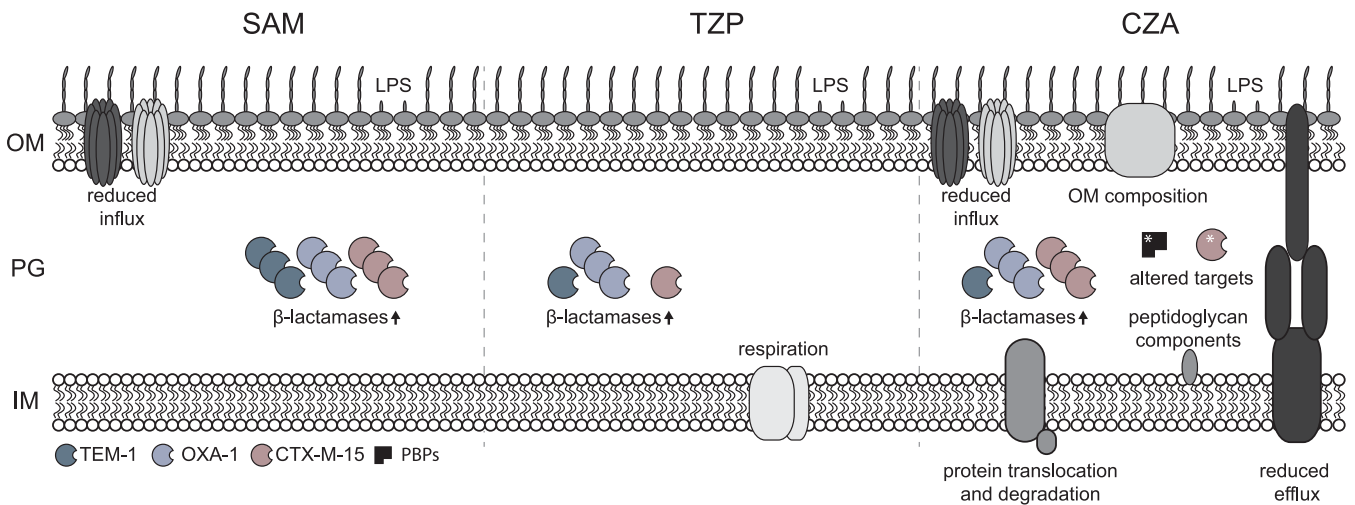


FIG 6 Overview of processes affected in achieving reduced susceptibility to β -lactams and β -lactamase inhibitors. The dominant mechanisms are illustrated for each combination. OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

we found a large number of mutations in genes involved in cell envelope synthesis and integrity at later stages of evolution. Antagonistic pleiotropic effects of the coproduction of different β -lactamases or combinations with other resistance mechanisms have, in view of the frequent occurrence of collateral sensitivity by *in vitro*-selected mutant CTX-M enzymes, been speculated to limit the *in vivo* selection of mutant enzymes resistant to inhibitors (37, 53). For combination therapy, β -lactamase enzyme mutations can be predicted to affect either the β -lactam or the inhibitor. The G238D substitution most likely interferes with the binding of avibactam since this mutation led to more susceptibility to ceftazidime alone. The P167T substitution has instead been observed to decrease susceptibility to ceftazidime and therefore also increases resistance to the CZA combination (52). Interestingly the second-residue exchange of one of the CZA mutants, N132K, is at an important site for both β -lactam and avibactam binding, creating a strong hydrogen bond to the substrate (54). This mutation could be a trade-off between being able to hydrolyze the β -lactam and avoiding strong binding of the β -lactamase inhibitor avibactam. Additional studies are needed to more exactly decipher how these mutations affect the catalytic potential of the enzyme.

A general trend for the evolved populations was a decrease in growth compared to the parental strain. This would potentially lead to lower competitive fitness under nonselective conditions. Amplifications of $bla_{CTX-M-15}$ and bla_{OXA-1} could be directly associated with an increased cost of gene expression since one particular mutant, DA61262, had a decreased growth rate of about 40%, without additional background mutations. We previously found that amplifications of this region of the resistance cassette confer a fitness cost to the cell and that this cost is coupled to the increased expression of β -lactamases (55, 56). However, amplifications could play a beneficial role as stepping-stones for the bacterium during selective circumstances due to their higher frequency of occurrence than point mutations. In addition, their inherent instability could rapidly revert the cost and thereby also the resistance phenotype and make the strain more competitive again when the selective pressure has been removed. This could result in the phenomenon called heteroresistance, which can be a complicating factor when testing susceptibility in clinical laboratories (57).

To conclude, we find that the combination of different β -lactamases and the amplification of one or several β -lactamase genes are very important features for increased resistance to β -lactam/ β -lactamase inhibitor combinations. The relatively high abundances of gene duplications in bacterial populations followed by additional amplifications let them act as stepping-stones toward higher-level, but rarer, resistance mutations. In addition, mutations in $bla_{CTX-M-15}$ alone could make the bacterium less susceptible to CZA, and this effect was strengthened by the amplification of the mutated allele in combination with reduced drug influx and mutations in other central cell functions. These findings corroborate previous

findings of the development of resistance of single β -lactamases to β -lactam/ β -lactamase inhibitor combinations and further outline the trajectories of the evolution of resistance against this important group of drugs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All constructed strains were derived from *Escherichia coli* MG1655 (see Table S1 in the supplemental material) and grown on Mueller-Hinton agar (MHA; Becton, Dickinson and Company) or in Mueller-Hinton broth (MHB; Becton, Dickinson and Company) with selection by trimethoprim (10 mg/L; Sigma-Aldrich) for the maintenance of the pUUH239.2 plasmid. The β -lactam antibiotics and β -lactamase inhibitors (Sigma-Aldrich) were used at clinical ratios of 2:1 for ampicillin-sulbactam, 8:1 for piperacillin-tazobactam, and 4:1 for ceftazidime-avibactam. Kanamycin (50 mg/L; Sigma-Aldrich) was used for the maintenance of pBAD18 vectors.

Susceptibility testing. Susceptibility testing was performed by broth microdilution according to EUCAST guidelines (58). For β -lactam/ β -lactamase inhibitor combinations, two different MICs were determined, the standard MIC according to EUCAST, with a variable concentration of the antibiotic and a fixed concentration of 4 mg/L of the inhibitor, and an MIC where the concentrations of both the antibiotic and inhibitor were changing but with fixed clinical ratios of 2:1 for SAM, 8:1 for TZP, and 4:1 for CZA, denoted MIC_{ratio} here. Since arabinose induction is negatively affected by glucose (59), MICs for strains with β -lactamases cloned on pBAD18 expression vectors were measured in tryptone broth at 37°C. Induction from pBAD18 was done by the addition of 0.05% L-arabinose, and 50 mg/L kanamycin was included to select for the plasmid.

Checkerboard susceptibility assay. Susceptibility to the β -lactam antibiotics at a wider range of β -lactamase inhibitor concentrations was evaluated with a checkerboard assay in 96-well plates. Checkerboard panels were prepared with 2-fold dilution series in MHB with final concentrations of 0.5 to 256 mg/L for ampicillin and piperacillin, 0.062 to 4 mg/L for ceftazidime, 2 to 128 mg/L for sulbactam, 1 to 64 mg/L for tazobactam, and 0.016 to 8 mg/L for avibactam. Cultures of DA5438 (*E. coli* MG1655) and DA63522 (*E. coli* MG1655/pUUH239.2) grown overnight were diluted in MHB and adjusted to a final inoculum of 5×10^5 CFU/mL. Plates were incubated at 37°C for 20 to 24 h. The experiments were performed with at least three biological replicates. *E. coli* ATCC 25922, *E. coli* ATCC 35218, and *K. pneumoniae* ATCC 700603 were used as quality control strains according to EUCAST guidelines (58).

In vitro selection of mutants with reduced susceptibility to combinations of β -lactam antibiotics and β -lactamase inhibitors. Single-step mutants with decreased susceptibility to the antibiotic-inhibitor combinations were selected in fluctuation assays by plating 1×10^7 and 1×10^8 CFU/mL onto plates containing $1\times$, $2\times$, and $4\times$ the MIC_{ratio} of the respective drug combination and incubated overnight at 37°C. The mutant frequency was calculated as r/Nt , where r is the number of mutants (CFU per milliliter) from the selective plate and Nt is the total number of viable cells (CFU per milliliter) from nonselective plates.

Continuous-evolution experiments were performed with eight independent lineages by passaging bacterial cultures at increasing concentrations of the antibiotic-inhibitor with a daily bottleneck of about 10^7 CFU (1 μ L of the culture grown overnight to 1 mL of fresh medium) for SAM and CZA and 10^6 CFU for TZP. The different bottleneck for TZP was due to observed inoculum effects at 10^7 CFU. Inocula were transferred to $1\times$, $2\times$, and $4\times$ MIC_{ratio} of the drug combination and incubated overnight. The culture with the highest concentration showing dense growth (visible by the naked eye as for broth microdilution susceptibility testing) was used for the next transfer at additional $1\times$, $2\times$, and $4\times$ increases of the concentration in which it was previously grown. Since the culture in the evolution experiment with CZA did not grow immediately at or above the initial MIC_{ratio}, these lineages were first passaged once at $1/2$ MIC_{ratio} before cycling at increasing concentrations.

Growth rate assay. Growth rates were measured with four biological replicates at 37°C in MHB using a BioscreenC MBR (Oy Growth Curves Ad Ltd.), taking measurements at an optical density at 600 nm (OD₆₀₀) every 4 min for 20 h. The maximum exponential growth rate was based on OD₆₀₀ values of between 0.02 and 0.08. The growth rates of the mutants were calculated relative to that of the parental strain.

Determination of β -lactamase transcription levels. The relative transcription levels of the β -lactamase genes *bla*_{TEM-1}, *bla*_{OXA-1}, and *bla*_{CTX-M-15} were measured for five mutants using real-time quantitative PCR (RT-qPCR). Cultures of three biological replicates grown overnight were diluted 1:500 in MHB and grown with antibiotic-inhibitor selection to an OD₆₀₀ of 0.15. Cultures were added at a 1:2 ratio to RNAprotect reagent (Qiagen). RNA extraction was performed using the RNeasy minikit (Qiagen) according to the manufacturer's instructions. DNA was removed from samples using the Turbo DNA-free kit (Ambion). A total of 500 ng RNA was used as the template for cDNA synthesis using a high-capacity reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. One hundred fifty microliters of water was added to the cDNA, and the sample was diluted (1:10, 1:100, and 1:1,000). The diluted cDNA was used as the template for RT-qPCR using the MiniOpticon real-time PCR system (Bio-Rad, Hercules, CA). The chromosomal housekeeping genes *hcaT* and *cysG* were used as references. Primers used for the β -lactamase and housekeeping genes are listed in Table S2. The transcription levels were calculated as $2^{\Delta C}$, where $\Delta C = C_T^{\text{ref}} - C_T^{\text{target gene}}$. The transcription levels of the mutants were calculated relative to those of the parental strain DA63522.

Whole-genome resequencing. Genomic DNA was obtained with the Epicentre MasterPure DNA purification kit (Illumina Inc.). Samples were prepared with $2\times$ 300-bp paired-end read lengths using the Nextera XT DNA library preparation kit (Illumina Inc.) according to the manufacturer's instructions. The DNA fragment size distribution was confirmed using the Agilent high-sensitivity D1000 ScreenTape system (Agilent Technologies). A MiSeq desktop sequencer was used according to the manufacturer's instructions (Illumina Inc.) to perform sequencing. The data were analyzed using the CLC Genomics Workbench version 21.1 (CLCbio, Qiagen). An in-house reference sequence of the parental strain, including the pUUH239.2 plasmid (GenBank accession number

NC_016966), was used for the reference assembly of sequencing reads from the isolated mutants. Single nucleotide polymorphisms (SNPs), structural variations, and indels were determined using the respective tools in CLC Genomics Workbench. All genetic changes were also manually inspected, and for rearrangements (deletions, duplications, and inversions), *de novo* assembly and reference coverage analyses were used to identify the respective changes and measure coverage for amplified regions. For population sequence analysis, a cutoff of 20% was set as the lowest detection level for genetic variations.

Data availability. Raw data from sequencing have been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA798964.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Research Council of Medicine and Health (2012-1511), the Carl Tryggers Stiftelse (CTS16:395), and the Åke Wibergs Stiftelse to LS.

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