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The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification

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When choosing antibiotics to treat bacterial infections, it is assumed that the susceptibility of the target bacteria to an antibiotic is reflected by laboratory estimates of the minimum inhibitory concentration (MIC) needed to prevent bacterial growth. A caveat of using MIC data for this purpose is heteroresistance, the presence of a resistant subpopulation in a main population of susceptible cells. We investigated the prevalence and mechanisms of heteroresistance in 41 clinical isolates of the pathogens *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* against 28 different antibiotics. For the 766 bacteria–antibiotic combinations tested, as much as 27.4% of the total was heteroresistant. Genetic analysis demonstrated that a majority of heteroresistance cases were unstable, with an increased resistance of the subpopulations resulting from spontaneous tandem amplifications, typically including known resistance genes. Using mathematical modelling, we show how heteroresistance in the parameter range estimated in this study can result in the failure of antibiotic treatment of infections with bacteria that are classified as antibiotic susceptible. The high prevalence of heteroresistance with the potential for treatment failure highlights the limitations of MIC as the sole criterion for susceptibility determinations. These results call for the development of facile and rapid protocols to identify heteroresistance in pathogens.

It is clear that genetically resistant bacteria have been, and will continue to be, a source of failure of antibiotic treatment and associated increased morbidity and mortality of infections[1,2]. In addition, host factors such as a reduced efficacy of the immune system, or acquired resistance by initially susceptible bacteria, may affect the rates at which pathogenic bacteria are cleared during antibiotic treatment. However, it is generally assumed that pathogens deemed susceptible to an antibiotic by the official minimum inhibitory concentration (MIC) breakpoint-based criterion can be effectively treated with that drug[3–5]. There is a caveat to this interpretation, since MICs are estimated under conditions that are optimal for the action of the drug, with low densities of bacteria growing exponentially in rich media and exposed to the antibiotic for a short period of time. However, when exposed to the antibiotic for longer periods, populations of pathogens classified as susceptible by this MIC criterion may, even in the absence of inherited resistance, replicate and cause treatment failure.

One reason for this limitation of MICs as the unique criterion for the susceptibility of bacteria to antibiotics is heteroresistance (HR), a phenotype in which the treated bacteria contain subpopulations with lower susceptibility to the antibiotic than the dominant population[6]. While densities of these minority populations are usually not sufficiently high to be reflected in the MIC, in the presence of antibiotic their frequencies increase and the MIC of the population at large approaches that of the least susceptible minority.

Although phenomenologically HR has been known for some time[6,7], it is rarely considered in clinical studies. Consequently, we know little about the prevalence of HR for most antibiotics among clinical bacteria, or its contribution to failed antibiotic treatment, even though some experimental animal and clinical studies implicate HR in such failures[8–13] and with it having being detected in clinical isolates of *Acinetobacter baumannii*[14], *Klebsiella pneumoniae*[15] *Escherichia coli*[16] and *Pseudomonas aeruginosa*[17] at high frequencies for carbapenems and colistin. We suggest there are two primary reasons for the relative dearth of studies estimating the prevalence of HR. One is the inconsistent definitions of HR and methodologies used to identify HR phenotypes[6]. The other is the difficulty of detecting HR by the methods currently employed by clinical laboratories to test for antibiotic susceptibility—for example, disk diffusion and broth microdilution[18,19]. Also, contributing to the dearth of consideration of HR as a potential source of treatment failure is the fact, that unlike inherited resistance, the mechanisms responsible for HR are largely unknown[8,20–24] and thereby the phenomenon is less widely accepted.

Here we present evidence for a high prevalence of HR among 41 clinical isolates of four different species of bacteria (*E. coli*, *K. pneumoniae*, *Salmonella enterica* serovar Typhimurium (hereafter referred to as *S.* Typhimurium) and *A. baumannii*) tested against 28 antibiotics. We demonstrate that more than half of the observed HRs in these clinical isolates can be attributed to unstable gene amplifications, commonly of known resistance genes. With the aid of mathematical modelling, we consider the pharmacodynamic properties of HR and how this unstable and transient resistance can lead to treatment failure. This study illustrates a major limitation of using current MIC estimates as the unique criterion for determining antibiotic susceptibility, and emphasizes the need to develop facile procedures to rapidly identify HR.

Results

Prevalence of HR in clinical isolates. We defined HR according to El-Halfawy and Valvano[6] as the presence of a subpopulation of cells capable of growing at concentrations of the antibiotic at least eightfold higher than the highest concentration that does not affect the replication of the dominant population (see Methods). We further

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restricted HR to the presence of resistant subpopulations at frequencies of 1×10^{-7} or higher, based on the fact that most mutational resistances occur at rates $<10^{-7}$ per cell per generation[25].

Based on this definition, we assayed 41 clinical isolates of *E. coli* ($n=11$), *K. pneumoniae* ($n=10$), *S.* Typhimurium ($n=10$) and *A. baumannii* ($n=10$) for HR against 28 different antibiotics covering all major classes, including clinically relevant drugs and those with defined breakpoints according to the European Committee on Antibiotic Susceptibility Testing (methodology summarized in Fig. 1 and presented in detail in Methods). The *A. baumannii* isolates had previously been described as HR to at least one antibiotic used in our study[9,20], but the *E. coli*, *K. pneumoniae* and *S.* Typhimurium isolates had not.

Based on our definition of HR (see above), we confirmed that 11.4% of the 766 bacteria–antibiotic combinations were HR (Fig. 2 and Supplementary Tables 1 and 2). Of the initial modified Etests (Epsilometer tests) showing potential HR, 37.9% were false positives because the phenotype could not be confirmed by population analysis profile (PAP) tests. Similarly, 14.7% of the modified Etests originally negative for HR were predicted to be false negatives according to subsequent tests. These results confirmed previous work showing that Etests are ineffective at detecting HR[18,19] (Fig. 2 and Supplementary Figs. 1 and 2). In summary, the screen revealed that up to 27.4% of the bacteria–antibiotic combinations examined (where the isolate was not fully resistant) showed HR (see Fig. 2 and Supplementary Table 3 for details of calculations). High frequencies of HR were observed for tetracycline, cephalosporins, carbapenems and trimethoprim (see Supplementary Notes and Supplementary Table 1), whereas we did not observe HR with quinolones, fluoroquinolones, erythromycin or rifampicin. Similarly, HR was not observed with polymyxin B and colistin, even though HR to both drugs has been described in these species[21,26,27] and was a frequent phenotype in previous studies with clinical isolates of *K. pneumoniae*[15,28] and *A. baumannii*[10,29]. The reason for the absence of HR for polymyxins in our study could be due to differences between our and previous studies in how HR is identified and/or the origin of the clinical strains. Only three out of eleven HR cases previously described for the *A. baumannii* isolates were detected as HR (DA33098 and DA33382 HR to amikacin and imipenem, respectively), or potentially HR (Supplementary Table 1).

Resistance is unstable and transient for the majority of identified HR cases. The phenotypic stability of resistant clones from the subpopulations of 34 confirmed cases of HR was tested. The terms resistant or resistance are used to describe decreased susceptibility, whether or not the MIC reached the clinically relevant breakpoint levels. For this test, the resistant subpopulations were grown for 40 generations in the absence of antibiotic pressure and tested for a decline in their levels of resistance. In four cases (12%) the resistance of the subpopulations was maintained, while in the remaining 30 cases (88%) the resistance was unstable and transient and the MIC fully or partially reverted back to the level of susceptibility of the parental isolate (Fig. 2 and Supplementary Table 1).

To determine the genetics behind the most common unstable HR phenotype, 18 of these HR cases were further analysed. For each case, whole-genome sequencing (WGS) was performed on the parental strain, a mutant from the resistant subpopulation and a revertant selected after growth in the absence of antibiotic. Mutants were selected at antibiotic concentrations where the frequency of the subpopulation with decreased susceptibility ranged from 10^{-4} to 10^{-7} (Supplementary Fig. 3 for PAP tests and Supplementary Fig. 4 for Etests). As described below, unstable HR resulted from either unstable genetic amplifications or other frequent mutational events, such as point mutations or small deletions (Tables 1 and 2).

Unstable HR linked to unstable tandem amplifications. In 11 out of 18 (61%) unstable HR cases, DNA amplifications were selected during growth under antibiotic pressure (Table 1). Conversely, during growth in the absence of drug, decreased copy number of the amplified units, deletion of the amplified region or loss of the plasmid carrying the amplified units characterized the revertants in which resistance was reduced or lost.

Tandem amplifications were observed for all four species and were located on both plasmids and chromosomes. The frequency of mutants with amplifications (at eightfold above the highest concentration not affecting growth of the dominant population) was 2.9×10^{-7} to 4.9×10^{-5} with a median of 4.9×10^{-6} (Supplementary Fig. 3 for PAP tests, Supplementary Fig. 4 for Etests). The copy number of the amplified units varied among the different isolates and antibiotics, ranging from 2.2× (DA35511) to 70× (DA38259). Similarly, the extent of reversion varied considerably after 40 generations in the absence of antibiotic pressure, ranging from revertants with only partially decreased copy number (for example, DA39607) to others losing the amplified units completely (for example, loss of plasmid in DA55765 or deletion of the amplified units in DA55787). The copy number of three plasmids of the IncF incompatibility group increased 1.6- to 3.0-fold during growth in the presence of gentamicin (DA55583 and DA55584) or netilmicin (DA38259), and decreased in the revertants following growth in the absence of drugs (Table 1). The WGS results were confirmed by qPCR for DA55583 (3.0-fold increase as measured by WGS and qPCR) and, to a lesser extent, for DA55584 (1.6- and 1.3-fold increase as measured by WGS and qPCR, respectively) and DA38259 (2.2- and 1.3-fold increase as measured by WGS and qPCR, respectively). No additional mutations were found, and the amplified units did not carry genes or loci known to be involved in copy-number regulation. The size of the amplified units ranged from 4.2 to 279.8 kilobase pairs (kbp), and most amplifications carried known genes involved in resistance towards the antibiotics or class of antibiotics used for selection (Table 1). As previously observed[30], amplification breakpoints were located within directly repeated sequences such as transposases or insertion sequences (IS6) for amplifications on plasmids, or 16/23S rRNA, insertion sequences (IS6) or *qacE* for amplifications on chromosomes (Supplementary Table 4). Furthermore, very complex sequential mutational events were detected in certain mutants and revertants (Supplementary Fig. 5), and the frequency of HR increased with the MIC of the main population (Supplementary Fig. 6), a possible side effect of the frequent implication in the HR phenotype of horizontally transferred resistance genes.

To confirm that amplification of resistance genes was responsible for the HR phenotypes, we performed two different tests. First, in the parental isolates we deleted the resistance genes that were amplified in the resistant subpopulations. In the parental strain DA34833, which had a $bla_{\text{CARB-2}}$ copy number of 1, MIC of 0.125 mg l^{-1} and a HR phenotype (to cefepime), deletion of the gene $bla_{\text{CARB-2}}$ resulted in loss of the HR phenotype and a reduction of MIC to 0.047 mg l^{-1}. Similarly, in the parental strain DA34827 which had a *tetA(G)* copy number of 1, MIC of 24 mg l^{-1} and a HR phenotype (to tetracycline), deletion of the *tetA(G)* gene resulted in loss of the HR phenotype and a reduction of MIC to 1.5 mg l^{-1}. These results confirm that HR is dependent on the presence of the respective functional resistance genes. Secondly, we overexpressed $bla_{\text{CARB-2}}$ (amplified in resistant mutant DA39369, HR to cefepime) and *aac(6′)–1b-cr* (amplified in resistant mutant DA38259, HR to netilmicin) by cloning them in a vector behind an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and expressing them in *E. coli* MG1655. As expected, resistance to cefepime and netilmicin increased with the amount of IPTG used for induction (Fig. 3) and converted the population to fully resistant. These results confirm that increasing expression levels of those genes, as would happen during amplification, mimics the increased resistance of the subpopulations.

To confirm that amplifications were present before selection in the presence of drugs and not induced by the antibiotics, we

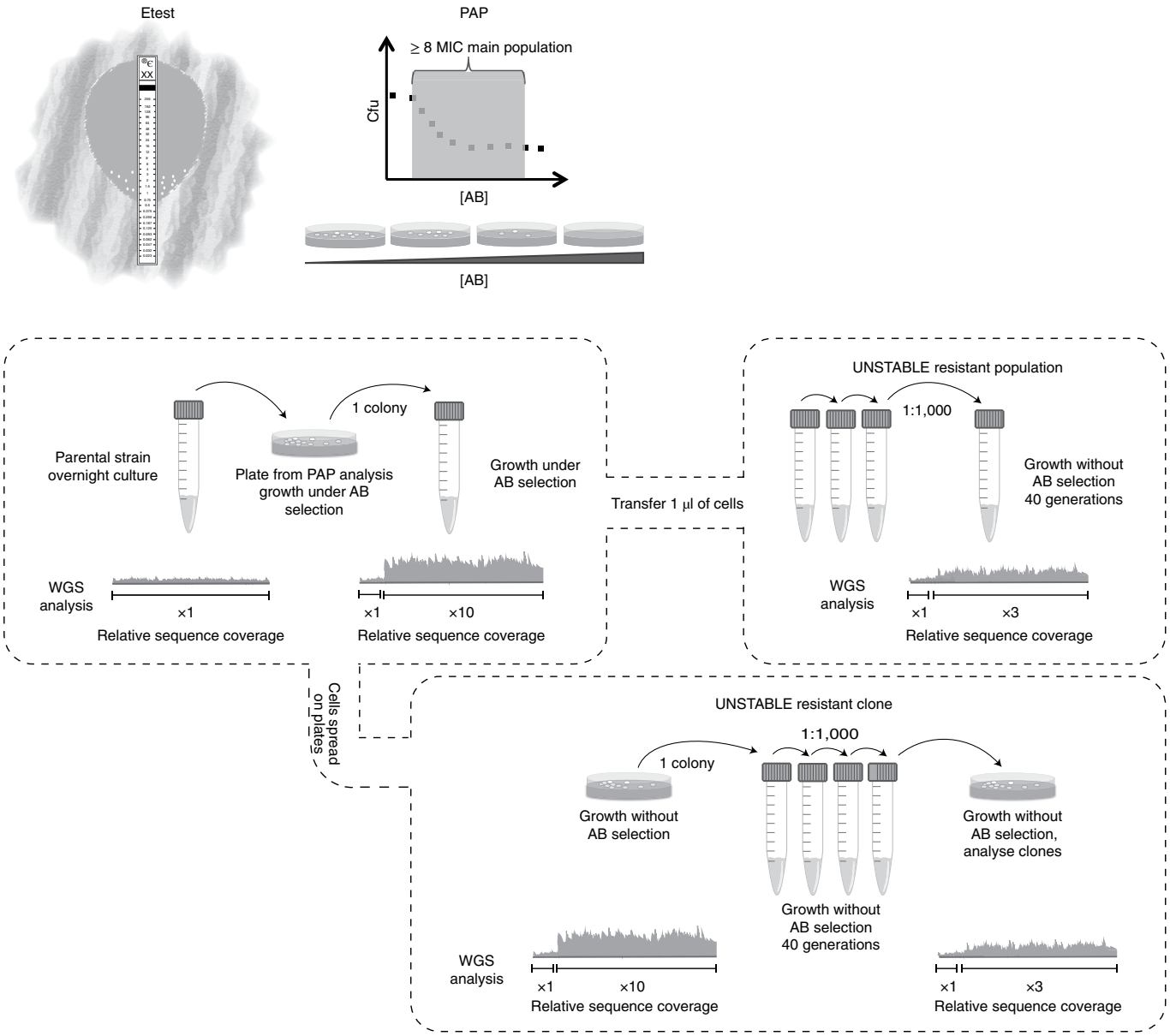


Fig. 1 | A schematic outline of methods used to determine HR frequency and the genetics behind unstable susceptibility/resistance to antibiotics among clinical *E. coli*, *S. Typhimurium*, *A. baumannii* and *K. pneumoniae* a, The 41 isolates (11 *E. coli*, 10 each of *S.* Typhimurium, *A. baumannii* and *K. pneumoniae*) were initially tested on 28 different antibiotics with one modified Etest (except for *A. baumannii*, in which 15 clinically relevant antibiotics were tested). Every strain exhibiting potential HR according to the initial Etest was tested with four additional modified Etests and four independent PAP tests to confirm HR, unless the MIC was above the [AB] that could be used in the PAP assay. **b**, A set of HR strains was tested for unstable HR. A resistant colony growing on one of the PAP test plates was re-streaked on the same concentration of antibiotics and subsequently grown overnight in broth containing the same antibiotic concentration. To test the instability of resistance, the strain was inoculated into Mueller–Hinton medium without antibiotic selection and cycled for 40 generations (1:1,000 daily dilution, growth for 4 days). To identify the genetics behind unstable HR in the parental strain, the resistant population (grown in broth in the presence of antibiotics), and the revertant population after 40 generations without antibiotic selection, were subjected to WGS and analysed by quantitative PCR (qPCR) to confirm the level of DNA amplification and/or plasmid copy number. To confirm that HR was present in the clonal population and did not result from the presence of different populations in the initial culture used for stability tests, four clones from the antibiotic-selected culture were isolated on a Mueller–Hinton plate. The MIC of each clone was determined by Etest, and the presence of mutations initially detected by WGS was confirmed by qPCR, PCR and sequencing. A correct clone was again subjected to WGS, inoculated into Mueller–Hinton broth without antibiotic selection and cycled for 40 generations. Ten clones from the last cycling step were isolated on a Mueller–Hinton plate and MIC was determined using a modified Etest. One or two clones with decreased resistance were once again subjected to WGS. Cfu, colony-forming units; [AB], antibiotic concentration; PAP, population analysis profile.

measured the spontaneous rates of duplications/amplifications of the amplified units found in DA36244 (parental strain DA34827) and DA39369 (parental strain DA34833) when the parental strains were grown in the absence of antibiotics. In both isolates, the frequencies measured were around 1×10^{-3} (Supplementary Fig. 7), indicating that duplications/amplifications were frequent and pre-existed in the absence of drugs.

Unstable HR linked to point mutations and small deletions. No amplifications were detected by WGS in 7 out of 18 clones with

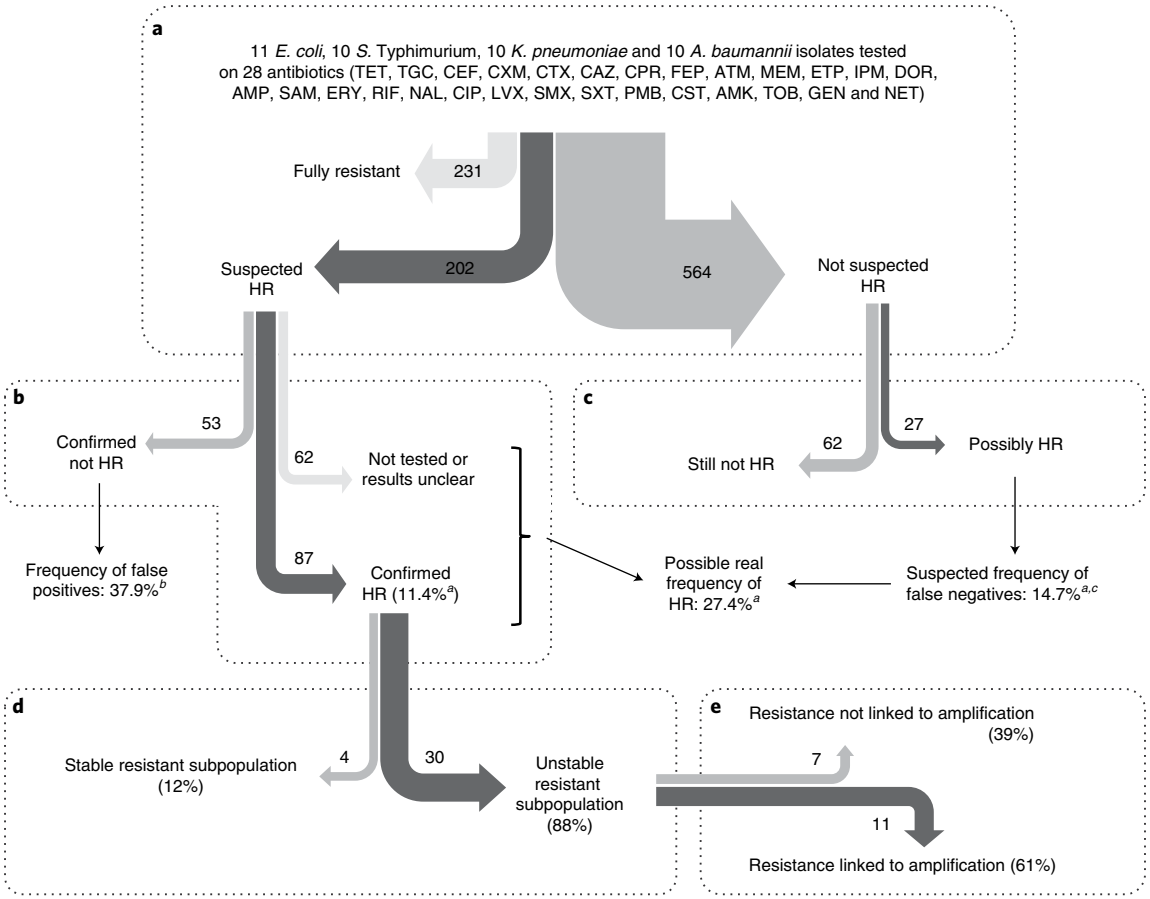
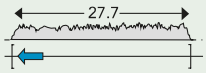
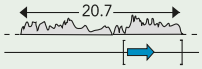
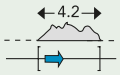
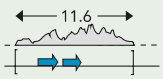
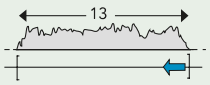
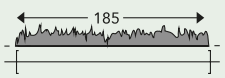
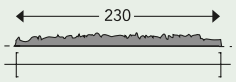
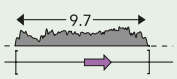
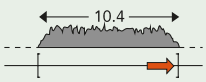


Fig. 2 | Summary of tests performed and main results. Antibiotics used include the classes tetracyclines (TET, tetracycline; TGC, tigecycline), cephalosporins (CEF, cephalothin; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CPR, cefpirome; FEP, cefepime), monobactams (ATM, aztreonam), carbapenems (MEM, meropenem; ETP, ertapenem; IPM, imipenem; DOR, doripenem), other beta-lactams (AMP, ampicillin; SAM, ampicillin/sulbactam), macrolides (ERY, erythromycin), rifamycins (RIF, rifampicin), quinolones (NAL, nalidixic acid), fluoroquinolones (CIP, ciprofloxacin; LVX, levofloxacin), sulfonamides (SMX, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole), polymyxins (PMB, polymyxin B; CST, colistin) and aminoglycosides (AMK, amikacin; TOB, tobramycin; GEN, gentamicin; NET, netilmicin). **a**, A single modified Etest was performed for each isolate–antibiotic combination (997 combinations). Fully resistant corresponds to tests where a dense population grew to the highest concentration of antibiotics on Etest. **b**, Four additional modified Etests and four PAP tests were performed on 184 cases of suspected HR. HR was confirmed when detected by PAP tests. **c**, Eighty-nine tests originally negative for HR following the first single Etest were retested with two additional modified Etests. **d**, The stability of the antibiotic resistance of the subpopulation was analysed for 34 cases of confirmed HR. **e**, The mutations involved in unstable HR were investigated by WGS for 18 cases. [a]Frequency among the original single Etests that did not show full resistance; [b]frequency among useable PAP tests results; [c]frequency adjusted according to results from PAP tests.

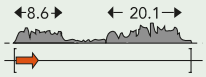
unstable resistance (Table 2). Instead, point mutations, insertion sequences (IS) insertions or small deletions were found. In contrast to what we observed with amplifications, these mutations did not affect known horizontally transferred resistance genes and were all chromosomally located. The frequency (at eightfold above the highest concentration that does not affect growth of the dominant population) of mutants with mutations was 4.7×10^{-7} to 9.7×10^{-5} with a median of 3.7×10^{-6} (Supplementary Fig. 3 for PAP tests, Supplementary Fig. 4 for Etests). Four mutants, selected in the presence of aminoglycosides, had mutations in genes or pathways known to result in a small colony variant phenotype and aminoglycoside resistance (*ubiJ* in DA52071 and *cydA* in DA55585, DA50426 and DA52072)[31,32], and grew ±30 to ±45% more slowly in Mueller–Hinton broth than their respective parental isolates, a characteristic of the small colony variant phenotype. Similarly the genes *fadB* and *mdh*, both mutated in DA51492 selected on amikacin, have been linked to aminoglycoside resistance/tolerance[33,34]. Other mutated genes (in DA52075 and DA55836) have so far not been associated with resistance to aminoglycosides or trimethoprim/sulfamethoxazole. When the mutants were grown in the absence of antibiotics for 40 generations, compensatory point mutations or amplifications increasing susceptibility were selected (Table 2). For DA52071 (*ubiJ* mutation) and DA55836 (*cpxA* mutation), compensation occurred by either amplification of *ubi* genes (DA53583) or loss of function mutations in *cpxR* of the *cpxA-cpxR* two-component system (DA56138), respectively. Because the in-frame small deletion in the sensor component *cpxA* was compensated by loss of function mutations in the transcriptional regulator *cpxR*, we speculate that the mutated CpxA might constantly activate CpxR, which would regulate expression of unknown gene(s) involved in trimethoprim/sulfamethoxazole resistance. CpxRA has not previously been associated with resistance to those antibiotics, but CpxR decreases susceptibility towards other antibiotics through activation of membrane pumps in *E. coli* and *P. aeruginosa*[35,36]. Both DA50426 and DA55585, which had mutations affecting *cydA*, were compensated by mutations in *arcA*, a gene coding for a known regulator of *cydA*[37]. Compensation in DA51492 selected on amikacin occurred through loss of function in *adeB*, a gene coding for a known multidrug pump involved in resistance to several antibiotics including aminoglycosides in *A. baumannii*[38]. Stochastic gene expression has

Table 1 | Unstable HR with DNA amplifications

Sample		Growth with selection		Growth without selection		Amplifications and antibiotic resistance genes (ResFinder) in the amplified region		
Strain **Sample type** **Antibiotic**	**MIC mg l^{-1} (HR)**	**Level of amplification WGS (qPCR)**	**MIC mg l^{-1} (HR)**	**Level of amplification WGS (qPCR)**	**MIC mg l^{-1} (HR)**	**Size (kb) of the amplified unit**	**Putative antibiotic resistance gene(s) involved in HR**	**Other antibiotic resistance genes**
E. coli DA33135 Population Tobramycin	3 (8)	**DA35498** 11.4x[b] [8.6x (9.2x)[c], *1.3x*[d]][e]	32 (64)	**DA35553** 3.4x [3.6x (2.5x), *0.9x*]	16 (128)		*aac(3)-Ild*	*tet*(A)[a] *sul1*, *sul2* *dfrA17* *aadA5* *mph*(A) *aph(6)-Id* *aph(3'')-Ib*
E. coli DA33137 Clone Gentamicin	16 (256)	**DA55584** 25.6x [16.1x, *1.6x*]	>256	**DA55769** 0.7x [0.9x, *0.7x*]	48 (>256)		*aac(3)-Ild*	*erm*(B) *dfrA17* *aadA5* *sul1* *mph*(A)
K. pneumoniae DA33140 Clone Gentamicin	24 (64)	**DA55583** 48x [16.2x, *3x*]	>256	**DA55765**, **DA55767** DA55765 loss of plasmid DA55767: 0.2x [0.8x, *0.3x*]	0.38 32 (>256)		*aac(3)-IIa*	None[f]
K. pneumoniae DA33140 Clone Tobramycin	16 (>256)	**DA55586** 3.1x [5.4x, *0.6x*]	>256	**DA55778**, **DA55780** DA55778 loss of plasmid DA55780: 2.7x [3.7x, *0.7x*]	0.5 96 (>256)		*aac(6')-Ib-ct* *aac(3)-IIa*	bla_{OXA-1} *catB4*
K. pneumoniae DA33141 Population Netilmicin	1 (2)	**DA38259** 69.9x [31.8x (29x), *2.2x*]	24	**DA38291** 13.8x [9.3x (5.2x),*1.5x*]	12		*aac(6')-Ib-cr*	bla_{OXA-1}[a] *tet*(A)[a] *catB4*
K. pneumoniae DA33145 Population Trimethoprim /sulfamethoxazole Two different mutants	0.38 (0.75)	**DA35510** 2.6x **DA35511** 2.2x	4 (16) 32	**DA35565** 1.1x **DA35566** 1.4x	0.38 (1.5) 0.38 (3)	 	None None	None[f] None[f]
***S.* Typhimurium** DA34827 Population Tetracycline	12 (32)	**DA36244** 23.9x (17.6x)	>256	**DA36372** 19.7x (13.3x)	96 (>256)		*tet*(A)	*floR* *aadA2*
***S.* Typhimurium** DA34833 Population Cefepime	0.19 (0.75)	**DA39369** 7.9x	1.5 (6)	**DA39607** 7.1x	0.75 (1.5)		bla_{CARB-2}	*tet*(A)[a] *floR*

continued

Table 1 | Unstable HR with DNA amplifications (Continued)

Sample		Growth with selection		Growth without selection		Amplifications and antibiotic resistance genes (ResFinder) in the amplified region		
Strain Sample type Antibiotic	MIC mg l^{-1} (HR)	Level of amplification WGS (qPCR)	MIC mg l^{-1} (HR)	Level of amplification WGS (qPCR)	MIC mg l^{-1} (HR)	Size (kb) of the amplified unit	Putative antibiotic resistance gene(s) involved in HR	Other antibiotic resistance genes
***S.* Typhimurium** DA34837 Clone Cephalothin	6 (12)	**DA55589** 5.1x	>32	**DA55787, DA55788** DA55787: ΔABR DA55788: 2.3x	2 16 (24)		*bla_{TEM-1B}*	*aph(6)-Id* *aph(3'')-Ib* *sul2* *tet*(B)
A. baumannii DA33382 Clone Trimethoprim/ Sulfamethoxazole	4 (>32)	**DA55753** 3.9x	>32	**DA56140** 1.1x	2 (3)		*sul1*	*aadA1* *aph(3')-Ic*[f] *aac(3)-Ia*

[a]Indicates genes that might be involved in other confirmed HR of the strain. [b]Total level of amplification, amplification copy number on plasmid × plasmid copy number. [c]Amplification copy number on the plasmid, and within parentheses level of amplification measured by qPCR. [d]In italic, change in plasmid copy number compared to the parental strain. [e]Within brackets, amplification copy number per plasmid followed by plasmid copy number change compared to parental in italic. [f]Presence of additional mutations that are identical in mutant and revertant (see Supplementary Table 4). [g]DA34827 and DA34833 contain the same plasmid (99% nucleotide identity). Level of HR within parentheses (mg l^{-1}) determined by modified Etest. Resistance for aminoglycosides (blue arrows), tetracyclines (purple arrow), beta-lactams (orange arrows) and sulfamethoxazole (yellow arrow). Plasmid DNA (light gray) and chromosomal DNA (dark gray). ΔABR, deletion of the amplified antibiotic resistance genes.

Table 2 | Unstable HR without DNA amplifications

Sample		Growth with selection		Growth without selection	
Strain sample type antibiotic	MIC mg l^{-1} (HR)	Mutations	MIC mg l^{-1} (HR)	Mutations	MIC mg l^{-1} (HR)
E. coli DA33133		**DA55585**		**DA55776**	
Clone	8 (24)	IS1 insertion in *cydA* promoter	96 (>256)[a]	IS1 insertion in *cydA* promoter	32 (>256)[b]
Netilmicin				ArcA A97V	
K. pneumoniae DA33141		**DA52071**		**DA53583**	
Population and clone	2	*ubiJ* Δ10 nt	24	*ubiJ* Δ10 nt	8
Amikacin				2 X amplification of ubi genes	
K. pneumoniae DA33144		**DA52072**		**DA53587**	
Clone	3	*cydA* Δ57 nt	16	*cydA* Δ57 nt	3 (16)
Amikacin				Point mutation upstream hypothetical gene	
Two different mutants		**DA52075**		**DA53588**	
		PTS glucose transporter subunit IIA Δ1 nt	16 (64)	PTS glucose transporter subunit IIA, Δ1 nt	4 (8)
				Crp G142D	
***S.* Typhimurium** DA34821		**DA50426**		**DA51303**	
Clone	1(12)	*cydA* Δ1 nt	48[c]	*cydA* Δ1 nt	8 (24)[d]
Tobramycin				ArcA R167C	
***S.* Typhimurium** DA34833		**DA55836**		**DA56138**	
Clone	0.19 (0.38)	*cpxA* Δ6 nt	2 (>32)	*cpxA* Δ6 nt	0.125
Trimethoprim/				CpxR Q146STOP	
Sulfamethoxazole				Invasin D369G	
A. baumannii DA33098		**DA51492**		**DA53749**	
Clone	8 (192)	FadB H192Y	>256	FadB H192Y	24 (>256)
Amikacin		mdh Δ9 nt		*mdh* Δ9 nt	
				IS4 insertion in *adeB*	

Δ, deletion. nt, nucleotide(s). Highest MIC level for the HR population is shown in parentheses. [a]Dense subpopulation 48–96 mg l^{-1} (Supplementary Figure 8). [b]Dense subpopulation 8–32 mg l^{-1} (Supplementary Figure 8). [c]Dense subpopulation 2–48 mg l^{-1} (Supplementary Figure 8). [d]Dense subpopulation 2–8 mg l^{-1} (Supplementary Figure 8).

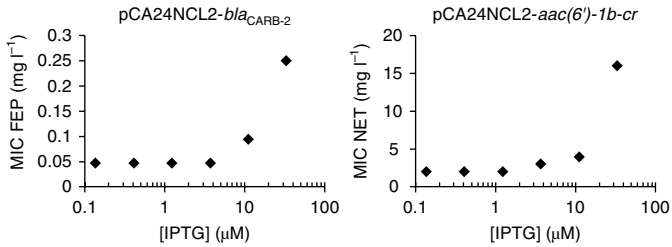


Fig. 3 | Overexpression of potential genes involved in unstable HR. Overexpression of resistance genes mimics the increase in resistance observed in strains with spontaneous genetic amplifications. Overexpression in *E. coli* MG1655 of genes cloned in plasmid pCA24NCL2 was achieved in the presence of increasing concentrations of IPTG, and MICs were determined by Etests. The experiments were performed twice independently with similar results. FEP, cefepime; NET, netilmicin.

been proposed as a possible mechanism for transient resistance and possibly HR[39,40], but is not likely to be a HR mechanism in our study.

While the process of reverting the copy number of amplified units leads to revertants with the same phenotype as the original isolate, the revertants of other mutation types described here were characterized by accumulation of compensatory mutations in new targets. Such revertants have a different genotype to the ancestral isolate, and some lost their HR phenotype (for example, DA53583 and DA56138) or displayed a different one (for example, DA53588 and DA55776) (Table 2 and Supplementary Fig. 8)[41].

HR can be due to several co-existing subpopulations. In the foregoing we have described the characteristics of pure single clones selected from resistant subpopulations. However, mutants isolated from the same subpopulation frequently displayed different MIC levels and stability of the resistance phenotypes (see, for example, Supplementary Fig. 9), indicating that several subpopulations characterized by their own mutation, frequency and phenotype might sometimes co-exist. Indeed, different mutations were found when two resistant clones were selected from DA33144 on amikacin (clones DA52072 and DA52075; Table 2) or from DA33145 on trimethoprim/sulfamethoxazole (clones DA35510 and DA35511; Table 1). This suggests that the analysis of isolated clones, as routinely performed in clinical laboratories, might not always adequately reflect the characteristics (for example, the resistance level and stability, or propensity to evolve higher resistance) of all bacteria present in a clinical sample.

The mechanistic instability and fitness cost of resistance mutations drive the reversion to susceptibility in the absence of antibiotic. Previous studies show that tandem gene amplifications are mechanistically highly unstable because of frequent unequal crossing over between homologous amplified copies[30,42–44]. Apart from the loss of the amplifications by crossing over, a reduction in copy number (and reduced resistance) in the population can also be driven by the fitness costs associated with the amplifications where, in the absence of antibiotic selection, cells lacking the amplifications grow faster than those with amplifications. Indeed, it is known that genetic amplifications confer a fitness cost and that they readily revert to single copy in the absence of selective pressure[45]. To determine whether reversion of a fitness cost is also important for instability in HR cases linked to other types of mutations, we compared the growth rate of parental isolates, mutants with decreased susceptibility and revertants (Supplementary Fig. 10a). In six cases, the resistant mutants had a high fitness cost (22–46% cost) that was partially or entirely compensated in the revertants (17–54% faster growth than the mutants from which they evolved). Only one unstable HR case (*A. baumannii* DA33098, selection on amikacin) differed, with both the resistant and compensated strains displaying 18–20% higher fitness than that of the parental strain, conceivably as the result of adaptation to the Mueller–Hinton medium.

In contrast to the unstable cases, resistant mutants from stable HRs had lower fitness costs (≤7%) that were not compensated following growth in the absence of selective pressure (Supplementary Fig. 10b). Similarly to what we described above, one unstable HR case (*A. baumannii* DA33112, selection on tobramycin) accumulated mutations that resulted in adaptation to the Mueller–Hinton medium. Because growth under antibiotic pressure was performed for about 60 generations, it is possible that some of the stable resistant mutants with mutations causing little to no effect on fitness evolved from mutants initially carrying unstable amplifications[46]. We did not test this hypothesis, as intermediate growth steps were not saved. Together, our results indicate that the mutations causing resistance in unstable HR cases are both costly and unstable, and that in the absence of antibiotic selection these factors together act as drivers for the population to revert to susceptibility.

Pharmacodynamics of HR and potential clinical implications. We mathematically modelled the pharmacodynamic properties of HR to explore the conditions under which HR can cause treatment failure. The structure of this model is similar to that in refs. [47–50] and is based on the unstable type of HR. For our numerical analysis of the properties of this model, we used parameter values estimated in the preceding experiments. For details of the modelling see Supplementary Methods. As shown by the results summarized in Supplementary Figs. 11 and 12, with realistic parameter values HR will often result in treatment failure.

Discussion

Heteroresistance has been studied since the 1940s, but only a few reports have explored its genetic basis or its involvement in treatment failure[7–13,21,24,51]. By using a strict definition of HR and a systematic approach, we found that HR was a common resistance phenotype among clinical isolates that would have been missed from standard estimates of MICs. Furthermore, unstable HR was frequent and was detected in 88% of HR cases, yielding an overall estimated frequency of unstable HR of 24.1% (88% of 27.4%) for the 766 bacteria–antibiotic combinations examined. Interestingly, for some classes of antibiotics where we did not detect HR (for example, quinolones/fluoroquinolones, rifampicin), resistance mainly evolves by target alteration. Thus, for those drugs the set of possible mutations increasing resistance might be too small for resistant mutants to appear at high frequency and HR to be observed. Inversely, for drugs where numerous mutations can cause resistance or where resistance genes can be amplified, the likelihood of observing HR might increase.

It is important to emphasize that the frequency of detected HR will depend not only on the chosen clinical strains and the antibiotics tested, but also on the detection method (for example, PAP test, Etests, disk diffusion or broth microdilution) and the HR definition chosen (for example, the cut-off resistance level chosen to identify a resistant subpopulation). For example, in our approach we might have missed true HR cases if HR was not suspected following the first Etest. This makes comparisons of studies difficult and underlines the importance of agreeing on a standard method and definition to identify HR in clinical isolates.

A summary of the mechanisms of HR described in our study is presented in Fig. 4. Of particular interest is the subtype described in Fig. 4a(i), which involves spontaneous unstable tandem amplifications, often of known resistance genes. This is the most common mechanism for HR that we observed in our study (61% of unstable HR cases) and also the most difficult to detect because of its high instability and transient nature. The high prevalence of this

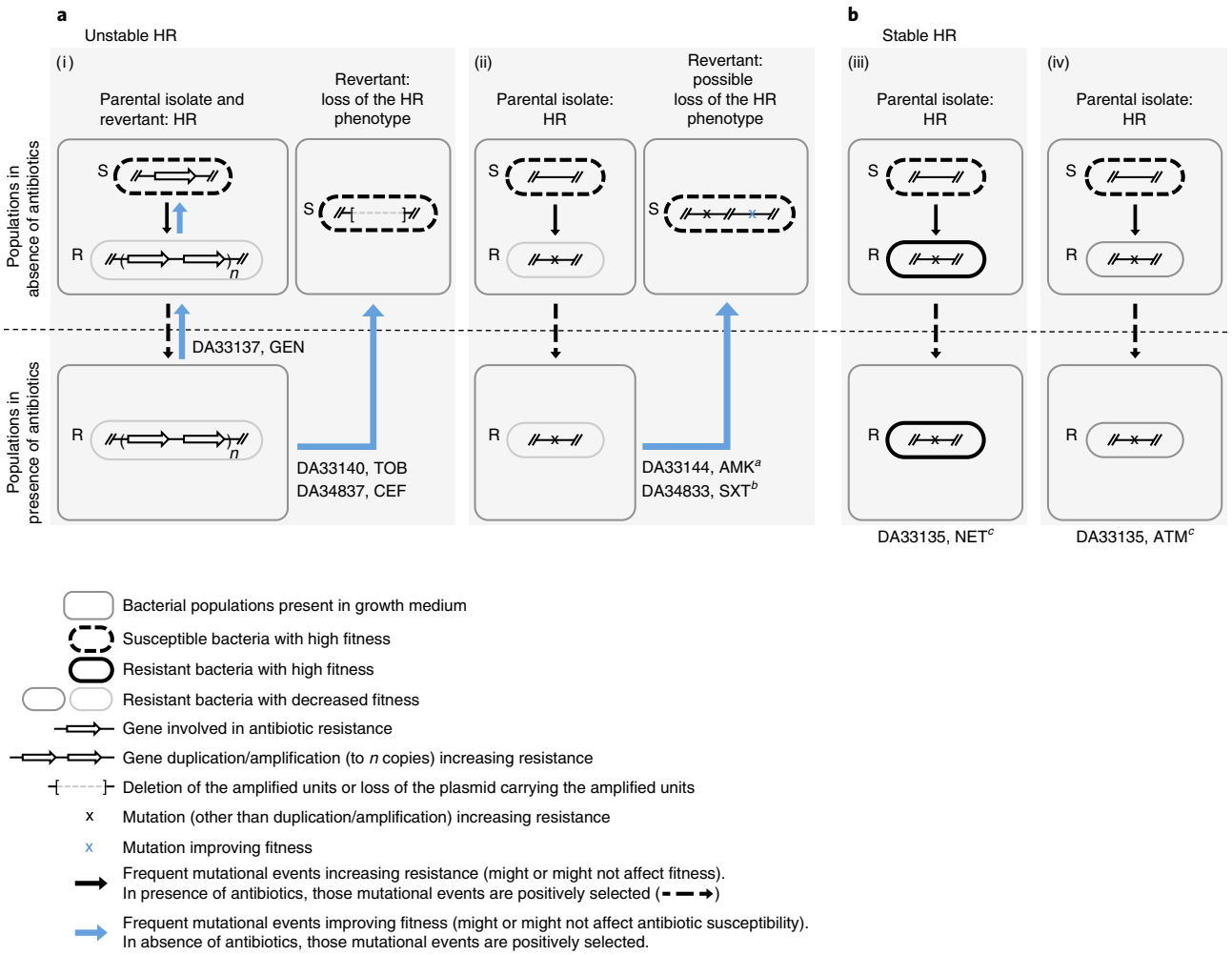


Fig. 4 | Molecular mechanisms of HR described in this work. Examples of stable and unstable HR cases are indicated. **a**, Unstable HR linked to genetic amplifications (i) or other types of mutations (ii). **b**, Stable HR. HR is stable if the mutation increasing resistance has no measurable effect on fitness (iii) or if the reversion rate or the effect on fitness is too low to allow revertants to take over rapidly during growth in the absence of antibiotic (iii, iv). [a]The revertant still had a HR phenotype; [b]The revertant lost the HR phenotype; [c]mutations were not determined, but their low (or no) cost and the inability of mutants to revert rapidly to the phenotype indicates that mutations, rather than amplifications, were probably involved. AMK, amikacin; ATM, aztreonam; CEF, cephalothin; GEN, gentamicin; NET, netilmicin; TOB, tobramycin; SXT, trimethoprim/sulfamethoxazole; S, sensitive; R, resistant.

HR mechanism can be rationalized by previous works which have shown that gene amplifications are very frequent in bacterial chromosomes (for example, the duplication frequency at different locations around the *Salmonella* chromosome varies between 3×10^{-2} and 6×10^{-5})[42], and that spontaneous duplications form and disappear at a high rate through homologous recombination between directly repeated sequences[30,42–44]. In addition, the involvement in resistance of amplifications of genes encoding efflux pumps, antibiotics targets or antibiotic-modifying or -degrading enzymes has been described[43]. However, the observation of gene amplifications being a general mechanism for unstable HR was unexpected and novel (previously described for only three cases[21,24,51]). The amplification-driven HR mechanism described in this study is expected to be dependent on the genetic background of the isolate, in that it requires (1) the presence of a gene that increases resistance when amplified; (2) the presence of direct repeat sequences around that gene to allow frequent spontaneous duplication; and (3) a low fitness cost of amplification. Underestimation of amplification-driven HR could be due partly to the difficulty in detecting amplifications and their intrinsic instability which, in the absence of antibiotic pressure, results in rapid de-amplification and reversion to the original genotype and susceptibility.

Because this study was carried out under laboratory conditions, we cannot directly evaluate the likelihood of HR leading to treatment failure of infections with pathogens that, based on their MICs, are assumed to be susceptible to the drug administered. On the other hand, our mathematical/simulation model suggests that, with the parameters in the range estimated and if the antibiotic is the sole mechanism controlling the infection[52], HR can lead to treatment failure.

In conclusion, our work highlights how HR is a challenge for the clinical microbiology laboratory, as well as for clinicians and their patients. Thus, because HR is so prevalent and difficult to diagnose with standard tests, it is likely that misdiagnosis of strains with a resistant subpopulation as fully susceptible is common, with potentially serious consequences in the form of treatment failure. In addition to the cautionary tale suggested by our modelling and by others[11], experiments with laboratory animals and *Enterobacter cloacae*[8], *K. pneumoniae*[12] and *Staphylococcus aureus*[13] also support the possibility that HR can indeed be responsible for failed treatment of infections with pathogens that, based on their MIC, would be classified as susceptible. The results of this study emphasize the need for well-controlled clinical studies on antibiotic-treated patients to estimate the relevant in vivo parameters (for example,

frequency and resistance level of the subpopulations) that are most likely to lead to treatment failure, and whether those parameters are patient-, disease-, bacterial species- and/or antibiotic-dependent. Our discovery of plasmid-associated HR mechanisms further accentuates the problem, since HR could spread horizontally between pathogens. Most importantly, to better arm ourselves against this insidious antibiotic resistance threat that is often undetected by established procedures, there is a need to develop broadly applicable methods to rapidly identify HR in pathogenic bacteria.

Methods

Bacteria, media and antibiotics. The isolates used in this work are described in Supplementary Table 4. Mueller–Hinton or cation-adjusted Mueller–Hinton II media (Difco, Becton Dickinson Company) were used as broth or in agar plates. The latter medium was used for growth in the presence of polymyxins (polymyxin B and colistin), while the former was used for all other antibiotics. Antibiotics were purchased from Sigma-Aldrich except for tigecycline, which was purchased from Apoteket as Tygacil (Wyeth). Whenever possible, antibiotic stock solutions were prepared fresh before PAP tests. All incubations were performed at 37 °C, under vigorous agitation for cultures in broth.

Isolation of clones from subpopulations with decreased susceptibility. Antibiotic resistant clones were selected from PAP test plates, re-isolated on similar antibiotic-containing plates and grown overnight in either of the broths described above in the presence of antibiotics (Fig. 1). The cultures were then used for modified Etests and stability of resistance tests, and an aliquot was stored at −80 °C in 10% dimethylsulfoxide. In parallel, 500 µl samples from the same cultures used for phenotypic testing were pelleted and stored at −20 °C for future DNA preparations and WGS. PAP tests and concentrations used for selection of mutants analysed by WGS are presented in Supplementary Table 4 and Supplementary Fig. 3. The frequency of cells growing at eightfold above the highest concentration that does not affect growth of the dominant population was calculated by dividing the population of bacteria growing in the presence of antibiotics by the total cell population without antibiotic selection.

Modified Etests. To increase the likelihood that resistant bacteria from the subpopulation are present and grow as isolated colonies in the inhibition zone of the main population, modified Etests with cell densities higher than usual were used[53]. Briefly, overnight bacterial growths in Mueller–Hinton broth were diluted 1:7 in phosphate buffered saline (PBS) medium, or colonies on Mueller–Hinton plates were resuspended in PBS (8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2HPO_4 and 0.24 g l^{-1} KH_2PO_4) or saline solution (9 g l^{-1} NaCl) to achieve 2 MacFarland units (approximatively 6×10^8 cells ml^{-1})[54]. The cell suspensions were evenly spread onto agar plates (with either medium described above) using sterile cotton swabs, and Etest strips (BioMerieux, or equivalent MIC Test Strips from Liofilchem or MIC evaluators from Oxoid) were applied. Plates were incubated for 48 h at 37 °C, and MICs were read after 48 h when HR was most visible. Two independent examiners (H.N. and K.H.) read each Etest, and HR was suspected when either isolated colonies grew in the inhibition zone or a halo of lower cell density was observed.

PAP tests. Bacterial strains were isolated on Mueller–Hinton agar plates, and four colonies were used to start four independent overnight cultures in 1 ml Mueller–Hinton broth (Fig. 1a). Each culture was then diluted 1:1,000 in PBS buffer, and 1 µl (about 4×10^3 cells) was used to inoculate 4 vials with 1 ml of Mueller–Hinton broth. The low cell count minimized the risk of the inoculum containing pre-existing resistant mutants. After overnight growth, 10^{-1} to 10^{-6} dilutions in PBS medium were prepared in a microtitre plate. Five microlitres from each dilution and from the undiluted culture were dropped on freshly prepared Mueller–Hinton or Mueller–Hinton II agar plates supplemented or not with increasing amounts of antibiotic (twofold increments). Plates were incubated overnight at 37 °C and colonies counted to determine the frequency of bacteria growing at each antibiotic concentration. To minimize the contribution of the density of the culture on the outcome of these experiments (the inoculum effect[55]), some PAP tests were performed again in a similar manner but the 5 µl drops were diluted into 50 µl of PBS before plating on larger surfaces (typically a whole agar plate) to lower cell density.

Stability of antibiotic resistance. For each test, two to four clones isolated from the subpopulation with decreased susceptibility (see above for selection) were used (Fig. 1b). After overnight growth of the selected clones in 1 ml of Mueller–Hinton broth supplemented with the same selection pressure (antibiotic concentration) as the plate from which the clone originated, MICs were determined by Etest. For each culture, a 500 µl aliquot was pelleted and stored at −20 °C for future DNA extraction and WGS, and another aliquot was stored at −80 °C in 10% dimethylsulfoxide. The cultures were then grown for four additional overnight periods (1 µl inoculated into 1 ml of Mueller–Hinton broth, ten generations per day) in the absence of antibiotics. The MICs after 40 generations in the absence of selective pressure were determined, and the resistance was deemed unstable if the MIC clearly decreased or reverted to that of the original parental isolate in at least one of the cultures. Aliquots from each culture were pelleted for future DNA extraction and WGS, or stored at −80 °C as described above.

Fitness cost measurements. Growth rates were analysed in a Bioscreen C apparatus (Oy Growth Curves Ab, Ltd) as follows. Four colonies for each test strain were used to inoculate four biological replicates in 1 ml of Mueller–Hinton broth. The overnight cultures were then diluted 1:1,000 in 1 ml of fresh Mueller–Hinton broth. The suspensions were used to start two to three 300 µl cultures (technical repeats) that were grown for 24 h at 37 °C in the Bioscreen apparatus. Absorbance ($A600_{nm}$) was measured every 4 min, with cultures shaken between measures. Absorbance values between 0.02 and 0.08, where growth is exponential, were used to calculate the maximum growth rate using BAT 2.0[56]. The relative growth rates were then normalized to that of the parental HR isolate, which was set to 1.

Cloning and overexpression of antibiotic resistance genes. The bla_{CARB-2} gene was PCR amplified from DA34833 using Phusion DNA polymerase (Thermo Fisher Scientific) and primers blaCARB_F2 and blaCARB_R2 (Supplementary Table 5). The PCR product was then purified using SureClean plus (Bioline). The purified PCR product and the IPTG-inducible plasmid pCA24NCL2[57] were digested using enzymes *Kpn*I and *Xma*JI (Thermo Scientific) and ligated together using T4 DNA ligase (GE Healthcare). Ligated products were cleaned using SureClean plus before electroporation into electrocompetent NEB 5-alpha *E. coli* (New England Biolabs). Electroporations were performed in 1 mm cuvettes using a Gene Pulser Xcell (Biorad) at 1.8 kV, 400 and 25 µF. Transformants were selected in the presence of chloramphenicol (12.5 mg l^{-1}) and verified by PCR and sequencing using primers pCA24N_check_F and pCA24N_check_R (Supplementary Table 5). The correct plasmid was extracted using the EZNA plasmid DNA Mini Kit I (Omega) and electroporated into electrocompetent *E. coli* MG1655. The pCA24NCL2-*aac(6′)−1b-cr* had been described previously[57].

Deletion of resistance genes. The bla_{CARB-2} gene and *tetR-tetA(G)* locus were deleted using the lambda Red method[58] as follows. The temperature-sensitive plasmid pSim10 (hygromycin resistance) expressing the lambda Red recombinase was electroporated into isolates DA34833 and DA34827, and transformants were selected in the presence of 50 mg l^{-1} hygromycin. The bla_{CARB-2} (DA34833) and *tetR-tetA(G)* (DA34827) genes were then replaced by the *aph(3′)* gene (for kanamycin resistance) amplified from plasmid pKD4[58] using Phusion DNA polymerase and primers delblacarB_DA34833_F + delblaCARB_DA34833_R and deltetRG_DA34827_F + deltetRG_DA34827_R, respectively (Supplementary Table 5), to construct strains DA58756 and DA58758. Transformants were selected at 37 °C in the presence of 50 mg l^{-1} kanamycin. Constructs were verified by PCR and sequencing using primers blaCARB_F1 + blaCARB_R1 and tetAG_F1 + tetAG_R1, respectively (Supplementary Table 5).

Measuring the frequency of spontaneous duplications/amplifications. The approach used is described in Supplementary Fig. 7. DA56659 (DA34833 carrying the lambda Red-inducible plasmid pSim10) and DA56661 (DA34827 carrying pSim10) were grown overnight in the presence of 50 mg l^{-1} hygromycin (for pSim10 selection) at 30 °C and used in lambda Red experiments as follows. A *dhfr* marker (trimethoprim resistance) was PCR amplified from the miniF-dhfr-orph11 plasmid (a plasmid from our laboratory) using Phusion DNA polymerase and primers floR_dhfr_LR_F + floR_dhfr_LR_R (Supplementary Table 5). Using the lambda Red method[58], the *dhfr* gene was introduced into the chloramphenicol resistance gene *floR* of DA56659 and DA56661; *floR* is present on the amplified regions previously found in mutants DA36244 (parental isolate, DA34827) and DA39369 (parental isolate, DA34833) (Supplementary Fig. 7a). Transformants were selected on Mueller–Hinton broth supplemented with 20 mg l^{-1} trimethoprim (total population of transformants) and on Mueller–Hinton broth supplemented with 20 mg l^{-1} trimethoprim and 24 mg l^{-1} chloramphenicol (population carrying duplications of *floR*: see Supplementary Fig. 7c). The frequency of spontaneous duplications/amplifications was calculated as the ratio of chloramphenicol + trimethoprim-resistant clones divided by the total number of trimethoprim-resistant clones. To verify that the selected transformants had the correct genotype, the presence of WT *floR* and/or the *floR:dhfr* allele was checked by PCR using primers floR_F1 + floR_R1 and floR_F1 + miniF_P17 (Supplementary Table 5 and Supplementary Fig. 7f). To ensure that lambda Red induction did not affect the frequency of spontaneous duplications/amplifications, we verified by PAP test that the frequency of tetracycline- and cefepime-resistant subpopulations was not affected (Supplementary Fig. 7d).

PacBio and Illumina WGS. *Samples.* Thirteen parental isolates (Tables 1 and 2) were analysed with PacBio and Illumina sequencing. Samples collected in the stability assay (500 µl frozen cell pellets) were analysed with Illumina sequencing. Either cell populations (pelleted samples) or pure clones were sequenced. To select pure clones, a resistant clone was re-isolated from the frozen stock and its MIC verified by Etest. An aliquot from the culture used for MIC determination was frozen at −80 °C in 10% dimethylsulfoxide, and 500 µl was pelleted and

stored at −20 °C for DNA preparation and WGS. The clone was then cycled for 40 generations in Mueller–Hinton broth without selection pressure, to revert the phenotype. Following growth, ten clones were isolated on a Mueller–Hinton plate and their MIC determined by Etest to verify reversion. An aliquot from the overnight culture of a revertant used for MIC determination was stored at −80 °C in 10% dimethylsulfoxide, and a sample of 500 µl was pelleted and stored at −20 °C for DNA preparation and WGS. *DNA extraction*. Genomic DNA from parental isolates (for PacBio and Illumina sequencing) was extracted from 3 ml of bacterial cultures in mid-exponential grown in Mueller–Hinton medium using the Genomic-tip 100/G kit (Qiagen) combined with the genomic DNA buffer kit (Qiagen) according to manufacturer's recommendation. For the mutants and revertants, DNA was extracted from the frozen cell pellets (for Illumina sequencing only) using the MasterPure Comp kit from Epicenter. DNA was resuspended in 10 mM Tris-HCl, pH 8.0, and concentrations were determined using the Nanodrop 1,000 (Themo Scientific) and Qubit 2.0 fluorometer (Invitrogen) apparatus. *PacBio sequencing*. Sequencing and assembly of the reads into contigs was performed at the National Genomics Infrastructure (SciLifeLab, Uppsala, Sweden). Sequences were trimmed (removal of overlapping sequences present at the ends of the contigs) and corrected with Illumina sequencing data. Corrected sequences were used as reference sequences for assembly of Illumina sequences of the mutants and revertants. *Illumina sequencing*. Illumina HiSeq2000 (500 base pairs paired-end libraries, ×50 coverage on average) was performed by BGI (Hongkong, China). Miseq (Illumina) sequencing was performed in house. For this, the libraries were prepared with the Nexteria XT DNA library preparation kit and the sequencing was done with a V3 600-cycle reagent cartridge. Sequencing was achieved with an average of at least ×30 coverage. *Sequence analysis*. Illumina sequences were mapped to the reference genomes using the CLC Genomic Workbench software (Qiagen). Mutations were detected with the CLC Genomic Workbench software using the basic variants detection, InDels and structural variants, and coverage analysis options. Potential phage infections or small plasmids missed by PacBio sequencing were detected by de novo assembly of unmapped reads. Potential antibiotic resistance genes were detected using ResFinder[59] and the default parameters (90% threshold, 60% minimum length). The level of DNA amplification was estimated by dividing the average sequence coverage of the amplification by the average sequence coverage of the entity where the amplification was located (plasmid or chromosome) in the WGS using the CLC Genomic Workbench. For calculation of the average sequence coverage of the plasmid/chromosome, the sequence of the amplification was removed from the WGS and the average coverage was recalculated. The sequence of the amplification (without the surrounding sequence) was used to recalculate the sequence coverage of the amplification. The increase/decrease in plasmid copy number was estimated from DNA coverage in the WGS using CLC by dividing the sequence coverage of the plasmid by the sequence coverage of the chromosome for each of the three strains in the set: the parental, the antibiotic selected and the strain without antibiotic selection. For the calculation of average sequence coverage of the plasmid, the DNA sequence of the amplification on the plasmid was removed from the WGS and the average sequence coverage was recalculated for the remaining plasmid sequence. The reference genomes submitted to the National Center for Biotechnology Information database were automatically annotated using the Prokaryotic Genome Annotation Pipeline at the National Center for Biotechnology Information.

qPCR experiments. The level of amplifications and plasmid copy numbers were verified by qPCR as follows. The qPCR mix consisted of 0.5 µl each of forward and reverse primers (10 µM), 5 µl of perfeCTa SYBR Green FastMix (Quanta biosciences) and 4 µl of the DNA samples used for WGS diluted 1:100, 1:1,000 or 1:10,000 in water. Amplifications were performed in an Eco Real-Time PCR system (Illumina). For each test, all pairs of primers used are described in Supplementary Table 5. Primer pairs in the target sequence (amplification or plasmid) and primer pairs in the control sequence (outside of the amplification, or on the chromosome) were used to calculate copy numbers of amplifications or plasmids. Copy numbers were calculated using the following formula: gene copy number $= 2^{CT(control) - CT(target)}$.

PCR and local sequencing. Genes to be sequenced were PCR amplified using the primers described in Supplementary Table 5 and the DreamTaq DNA polymerase master mix (Thermo Scientific). Products were purified using the GeneJET gel extraction kit (Thermo Scientific) and sequenced at Eurofins Genomics.

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

Data availability

Chromosomes and plasmids are deposited at NCBI under the following accession numbers: DA33098 (CP029569–CP029573), DA33133 (CP029574 and CP029575), DA33135 (CP029576–CP029578), DA33137 (CP029579–CP029581), DA33140 (CP029582–CP029586), DA33141 (CP029587–CP029589), DA33144 (CP029590–CP029592), DA33145 (CP029597–CP029599), DA33382 (CP030106–CP030109), DA34821 (CP029567), DA34827 (CP029593 and CP029594), DA34833 (CP029595 and CP029596) and DA34837 (CP029568). Additional data supporting the findings of this study, such as raw data and bacterial strains, are available upon request.

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Author contributions

H.N., K.H. and D.I.A. designed the study. H.N. and K.H. performed the experiments and B.R.L. the mathematical modelling. H.N., K.H., B.R.L. and D.I.A. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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