Received: 29 March 2022 | Accepted: 10 September 2022

DOI: 10.1111/1462-2920.16206

RESEARCH ARTICLE

environmental microbiology Applied Microbiology International

Antibiotic concentrations in raw hospital wastewater surpass minimal selective and minimum inhibitory concentrations of resistant *Acinetobacter baylyi* strains

Dominik Schuster[1] | **Katharina Axtmann**[1] | **Niklas Holstein**[1] | **Carsten Felder**[2] | **Alex Voigt**[2] | **Harald Färber**[2] | **Patrick Ciorba**[1] | **Christiane Szekat**[1] | **Anna Schallenberg**[1] | **Matthias Böckmann**[3] | **Christiane Zarfl**[3] | **Claudio Neidhöfer**[1] | **Kornelia Smalla**[4] | **Martin Exner**[2] | **Gabriele Bierbaum**[1]

[1]Institute of Medical Microbiology, Immunology and Parasitology, University Hospital Bonn, Bonn, Germany

[2]Institute for Hygiene and Public Health, University Hospital Bonn, Bonn, Germany

[3]Environmental Systems Analysis, Eberhard Karls University of Tuebingen, Tuebingen, Germany

[4]Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany

Correspondence
Gabriele Bierbaum, Institute of Medical Microbiology, Immunology and Parasitology, University Hospital Bonn, Venusberg-Campus 1, 53127 Bonn, Germany.
Email: g.bierbaum@uni-bonn.de

Funding information
Bundesministerium für Bildung und Forschung, Grant/Award Number: FKZ 02WRS1377; Deutsche Forschungsgemeinschaft, Grant/Award Number: 431531292

Abstract

Antibiotics are essential for modern medicine, they are employed frequently in hospitals and, therefore, present in hospital wastewater. Even in concentrations, that are lower than the minimum inhibitory concentrations (MICs) of susceptible bacteria, antibiotics may exert an influence and select resistant bacteria, if they exceed the MSCs (minimal selective concentrations) of resistant strains. Here, we compare the MSCs of fluorescently labelled *Acinetobacter baylyi* strains harboring spontaneous resistance mutations or a resistance plasmid with antibiotic concentrations determined in hospital wastewater. Low MSCs in the μg/L range were measured for the quinolone ciprofloxacin (17 μg/L) and for the carbapenem meropenem (30 μg/L). A 24 h continuous analysis of hospital wastewater showed daily fluctuations of the concentrations of these antibiotics with distinctive peaks at 7–8 p.m. and 5–6 a.m. The meropenem concentrations were always above the MSC and MIC values of *A. baylyi*. In addition, the ciprofloxacin concentrations were in the range of the lowest MSC for about half the time. These results explain the abundance of strains with meropenem and ciprofloxacin resistance in hospital wastewater and drains.

Originality-Significance Statement: In previous studies, we had isolated a great variety of multi-resistant bacteria (simultaneously resistant to the critically important antibiotics 3rd gen. cephalosporins, carbapenems, piperacillin/tazobactam, and ciprofloxacin) from the drains and the wastewater of a maximum care hospital. These bacteria reach the environment, since they are still detected in the effluent of the wastewater treatment plant and they cause infections that are very difficult to treat. So far, it has been assumed that good hygienic measures should be able to avoid nosocomial infections of patients with such bacteria. However, the key aspect of this manuscript is that fluctuating high antibiotic concentrations of meropenem, ciprofloxacin and piperacillin are present in hospital wastewater and that these antibiotic concentrations reach or surpass the minimal selective concentrations of the model organism Acinetobacter baylyi. In conclusion, the colonization of hospital drains and wastewater pipes with multi-resistant bacteria is probably driven by the high antibiotic concentrations in the wastewater. These results are very important, since the antibiotic concentrations and the resulting selective pressure will complicate attempts to such remove the bacteria from the drains and wastewater pipes by cleaning or disinfection measures.

INTRODUCTION

Antibiotics play an essential role in modern medicine, they enable therapy of infections as well as surgical and cancer treatment. In Europe, antibiotic consumption in 2019 has amounted to 19.4 defined daily doses per 1000 inhabitants per day (European Centre for Disease Prevention and Control 2020), which are partially released into the wastewater system after use, since most antibiotics are not fully metabolized. For example, 70% of meropenem (Zhanel *et al*. 2007), 56 to 73% of piperacillin (Tjandramaga *et al*. 1978) and 65% of ciprofloxacin (Rohwedder *et al*. 1990) are excreted renally. In addition, pharmaceutical production facilities discharge antibiotics and agriculture introduces antibiotic residues with organic fertilizers into the soil. In contrast to β-lactams (e.g., carbapenems, 3rd generation cephalosporins), that are quickly degraded in wastewater and soil (Ribeiro *et al*. 2018), quinolones (for instance ciprofloxacin), sulfonamides (such as sulfamethoxazole and sulfadiazine), trimethoprim, MLS_B antibiotics (clindamycin, erythromycin, azithromycin, clarithromycin, lincomycin, etc.) and disinfectants (e.g., quaternary ammonium compounds) are persistent enough to reach the environment (Lesser *et al*. 2018; Östman *et al*. 2017). At the beginning of a wastewater system, for example, in the drains of hospital showers and toilets, antibiotic concentrations may be rather high and reach the mg/L range (Voigt *et al*. 2019). In contrast, the concentrations that reach the environment are in the ng/L or low μg/L range (Michael *et al*. 2013). Therefore, environmental concentrations are lower than most minimal inhibitory concentrations (MICs) of susceptible bacteria, which are typically between 10 μg/L and 10 mg/L (MIC distributions are available from the EUCAST database, https://www.eucast.org/ [EUCAST 2022]).

So far, there is little knowledge on the impact of low concentrations of antibiotics on environmental bacteria. However, in laboratory competition experiments antibiotic resistant bacteria were selected by very low levels of antibiotics, which can be more than 100 fold below the MIC (Gullberg *et al*. 2011). Susceptible bacteria show a slower growth in the presence of subinhibitory antibiotic concentrations. Their growth rate is decreased by partial inhibition of the essential enzymatic process that is targeted by the antibiotic. Resistant strains, which harbor an antibiotic resistance gene or a mutation in the antibiotic target, are not affected at this concentration and grow faster than susceptible strains. However, resistance often causes fitness costs (Andersson and Hughes 2010), because a large plasmid must be replicated, resistance proteins must be expressed, transport is limited or the bacteria have to cope with the side effects of mutations in target or regulator genes. These fitness costs limit the growth rate of resistant strains at all antibiotic concentrations (Andersson and Hughes 2010). Hence, a susceptible strain will grow faster than the resistant strain in the absence of or at very low concentrations of the antibiotic. In contrast, the resistant strain will grow more slowly at all concentrations. However, resistant strains will be able to keep on growing, when antibiotics slow down the growth of the susceptible strain. The antibiotic concentration that results in identical growth rates of the resistant and susceptible strain is called minimal selective concentration (MSC), i.e., the MSC is the threshold concentration above which a resistant strain will grow faster than a susceptible strain (Gullberg *et al*. 2011; Khan *et al*. 2017). In addition, especially with sub-MIC concentrations, selection of resistant strains harboring point mutations is favored (Hughes and Andersson 2012).

Experimental MSCs for bacterial species have been published for a number of systems, however, with a strong focus on Enterobacterales, e.g., for *E. coli* and *S. enterica* harboring resistance genes or chromosomal mutations (Gullberg *et al*. 2011; Klümper *et al*. 2019), for the evolution of resistance in mutator strains of *S. enterica* (Wistrand-Yuen *et al*. 2018), horizontal gene transfer into *E. coli* (Jutkina *et al*. 2016), or with a focus on complex communities, e.g., the selection of resistance genes in wastewater communities (Lundström *et al*. 2016; Kraupner *et al*. 2018; Murray *et al*. 2018; Stanton *et al*. 2020). So far, to our knowledge, there is no experimental system that examines the effects of low antibiotic concentrations on species of other bacterial orders. Here, especially environmental bacteria that are able to proliferate in the environment and are associated with wastewater, like *Acinetobacter* species, are interesting. For example, in soil irrigated with wastewater, Gammaproteobacteria, among them *Acinetobacter* strains, are enriched (Broszat *et al*. 2014) as well as in treated sewage sludge (Wolters *et al*. 2022) and, on the other hand, strains affiliated to this genus were reported from vegetables, meat and livestock (Al Atrouni *et al*. 2016). In hospitals, several *Acinetobacter* species (*A. baumannii*, *A. pittii*, *A. lwoffii*, etc.) cause nosocomial infections with a high mortality rate in immunocompromised patients, which is why carbapenem-resistant *Acinetobacter* strains are now among the most problematic pathogens of multidrug-resistant or even pan-resistant infections (Wong *et al*. 2016).

Acinetobacter baylyi is an environmental bacterium that has been isolated from activated sludge of a wastewater treatment plant (Carr *et al*. 2003) and has been proposed as a model system for the genus *Acinetobacter* (Elliott and Neidle 2011; Barbe *et al*. 2004). *A. baylyi* employs a type VI secretion system for killing competing bacteria and takes up the DNA of its prey by competence transformation (Lin *et al*. 2019) which makes it an ideal organism to study selection and evolution of resistant strains in wastewater-associated environments. In addition, because of its ability to

become competent, *A. baylyi* is amenable to genetic manipulation at the S1 level (Juni and Janik 1969).

In a previous study, we had shown that a surprisingly high diversity of bacterial strains (belonging to the orders Enterobacterales and Pseudomonadales) with simultaneous resistance to ciprofloxacin, carbapenems, 3rd generation cephalosporins, and piperacillin/tazobactam was present in the wastewater of a maximum care hospital (Kehl *et al*. 2022; Sib *et al*. 2020). Such bacteria were also found repeatedly in the biofilms of the drains in the patients rooms (Kehl *et al*. 2022; Sib *et al*. 2019). In order to determine whether the antibiotics present in hospital wastewater might favor the survival and/or growth of these antibiotic resistant strains, we have constructed fluorescently labelled *A. baylyi* strains for the measurement of MSC values employing competition experiments. Here we compare their MSCs to antibiotic concentrations in raw hospital wastewater of a maximum care hospital.

RESULTS

Construction of plasmid bearing strain pairs

According to analysis with the program ResFinder 3.2, *A. baylyi* BD413 is free of exogenously acquired resistance genes. However, our testing showed resistance to cefotaxime and temocillin (see Table 1), and in fact, *A. baylyi* harbors the intrinsic AmpC β-lactamase (ADC-8) (Beceiro *et al*. 2007); the presence of such enzymes is typical for this genus, as also shown by bla_{ADC} in *A. baumannii* (Karah *et al*. 2017). The fluorescently labelled strains (harboring *gfp* or *mCherry*) were selected using a spectinomycin cassette that had been inserted downstream of the gene for the fluorescent protein and were named *A. baylyi* BD413 GFP and *A. baylyi* BD413 mCherry (**Figure S1**).

Both strains were transformed by natural competence with the 58 kb low GC-resistance plasmid pHHV216 (Heuer *et al*. 2009), since attempts at conjugation failed repeatedly. The plasmid pHHV216 confers resistance to sulfonamides, (e.g., sulfadiazine and sulfamethoxazole; *sul2*), tetracycline (*tetH*), chloramphenicol (*florR*), and streptomycin (*strA* and *strB*)). Growth assays showed that the growth rate was decreased in both plasmid-harboring strains compared to their parent strains. However, there were no differences between the strain expressing GFP or mCherry transformed with pHHV216 (Figure 1).

Selection of spontaneous mutants

After plating on Chromagar™ ESBL, two resistant isolates (*A. baylyi* BD413 mCherry 652, and *A. baylyi* BD413 mCherry 777) were isolated. Full genome sequencing and subsequent comparison with the published genome sequence of *A. baylyi* BD413 showed that the strains had acquired resistance through point mutations or a deletion (Table 2). In isolate *A. baylyi* BD413 mCherry 777, the gene *bfmS,* that encodes a sensor histidine kinase, was truncated by a stop codon and the mutant protein possessed only 478 amino acids instead of a 546 amino acids. Since the ATP binding site of these kinases (the HATPase_c domain) is located in the C-terminus of the protein (aa 428–532 according to the annotation in NCBI), BmfS was most probably inactivated. Another mutation of this strain was an exchange of leucine to serine in position 212 in ACIAD1367, annotated as a TetR/AcrR regulator. Blast comparisons showed that it is the homologue of AdeN, a repressor that inhibits the transcription of the RND (Resistance-nodulation-division) transporter AdeIJK in *A. baumannii* (Rosenfeld *et al*. 2012). The second isolate, *A. baylyi* BD413 mCherry 652 harbored a large deletion comprising the full AdeN regulator (ACIAD1367) and adjacent genes, stressing the importance of this repressor of the AdeIJK efflux pump (Table 2).

Growth rates

In growth assays, the effects of the deletion in *A. baylyi* BD413 mCherry 652 and the large plasmid pHHV216 on the growth rate were clearly visible, just as a strong effect of the mutations in *A. baylyi* BD413 mCherry 777. This strain showed a considerably lower the growth rate than the original strain and the plasmid-bearing variants indicating a high fitness cost (Figure 1).

Minimal inhibitory concentrations against antibiotics and heavy metals

As expected the MICs against the antibiotics covered by the plasmid were elevated in the strains harboring pHHV216, since the plasmid confers resistance to chloramphenicol, sulfonamides, streptomycin, and tetracycline (Jechalke *et al*. 2013). The spontaneous mutants showed increased MICs for several antibiotics, however, without displaying clinically relevant resistance according to the clinical EUCAST *Acinetobacter* spec. breakpoints for ciprofloxacin, meropenem, imipenem, colistin, and sulfamethoxazole/trimethoprim. The MICs of *A. baylyi* BD413 mCherry 777 showed a higher resistance to nearly all β-lactams and to ciprofloxacin, benzalkonium chloride, sulfamethoxazole, and clindamycin (see Table 1). EUCAST does not define breakpoints for cephalosporins and β-lactams for *Acinetobacter* species, since the clinically relevant *A. baumannii* is considered intrinsically resistant. However, using the non-species related breakpoint table

TABLE 1 The minimum inhibitory concentrations (MICs) of *A. baylyi* BD413 and its variants. EUCAST clinical breakpoints for *Acinetobacter* or values from the PK PD table are listed in the last two columns (EUCAST 2022) and MICs that indicate clinical resistance according to EUCAST have been marked in bold.

MIC [mg/L] (24 h)	wildtype	GFP/ mCherry	GFP/ mCherry	mCherry	mCherry	EUCAST *Aci.* R>	EUCAST PK PD R>
Antibiotic agent			pHHV216	652	777		
Plasmid encoded							
Chloramphenicol	≤8	≤8	>16	16	≤8	-*	-
Tetracycline	0.5	0.5	256	8	8	-	-
Sulfadiazine	8	8	512	16	128	-	-
Sulfamethoxazole	3	3	>256	16	128	-	-
Others							
Ciprofloxacin	0.02	0.02	0.039	0.313	0.313	1	
Levofloxacin	0.02	0.02/0.039	0.039	0.313	0.313	1	
Amikacin	≤4	≤4	≤4	≤4	≤4	8	
Colistin	≤1	≤1	≤1	≤1	≤1	2	
Fosfomycin	32	32	≤16	64	16	-	8 (oral)
Tigecycline	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	-	0.5
Trimethoprim/ Sulfamethoxazole	≤1/19	≤1/19	≤1/19	≤1/19	2/38	4/76	
Piperacillin	≤8	≤8	≤8	16	**>16**	-	16
Piperacillin/Tazobactam	≤4/4	≤4/4	4/4	8/4	**>64/4**	-	16/4
Cefotaxime	**>2**	**>2**	≤1	**>2**	**>2**	-	2
Ceftazidime	4	4	≤1	**16**	**>128**	-	8
Ceftazidime/Avibactam	4/4	4/4	≤1/4	**16/4**	**>16/4**	-	8/4
Ceftolozane/Tazobactam	≤1/4	≤1/4	≤1/4	2/4	**>8/4**	-	4/4
Imipenem	1	1	≤1	≤1	≤1	4	
Meropenem	≤0.125	≤0.125	≤0.125	0.5	2	8	
Temocillin	128	128	<32	>128	>128	-	-
Clindamycin	8	8	8	64	64	-	-
Erythromycin	3	3	3	16	8	-	-
Benzalkonium chloride	4	4	4	8	8	-	-
Heavy metals							
$Cu(II)SO_4$	128	128	>256	128	>256	-	-
$ZNSO_4*7H_2O$	64	64	128	128	128	-	-
$Pb_3(C_6H_5O_7)_2*3H_2O$	>512	>512	>512	>512	>512	-	-

*- value not defined.

(PK-PD), *A. baylyi* BD413 mCherry 777 had acquired full resistance to piperacillin, piperacillin/tazobactam, ceftazidime, ceftazdime/avibactam, and ceftozolane/tazobactam. *A. baylyi* BD413 mCherry 652 had reached resistance to ceftazidime and ceftazidime/avibactam. The wildtype strain and tested mutants were relatively resistant against heavy metals (Table 1).

Competition experiments

Using the strain pairs that carry the plasmid pHHV216 (*A. baylyi* BD413 GFP (susceptible) versus *A. baylyi* BD413 mCherry pHHV216 (resistant) and *A. baylyi* BD413 GFP pHHV216 (resistant) versus *A. baylyi* BD413 mCherry (susceptible)), or the mutant strains, MSC values were determined as shown in Figure 2, Table 3 and supplemental **Figures S2–S6**. A possible transfer of pHHV216 from the resistant to the susceptible strain during the experiments was monitored by plating aliquots of the mixed cultures after incubation on agar plates containing sulfadiazine, streptomycin, and tetracycline. In these controls, colonies of the susceptible plasmid-free strain were never detected and, therefore, plasmid transfer did not seem to be occurring. The MSCs of the spontaneous mutant strains (*A. baylyi* BD413 mCherry 652 and *A. baylyi* BD413 mCherry 777) are also shown in Table 3. A comparison

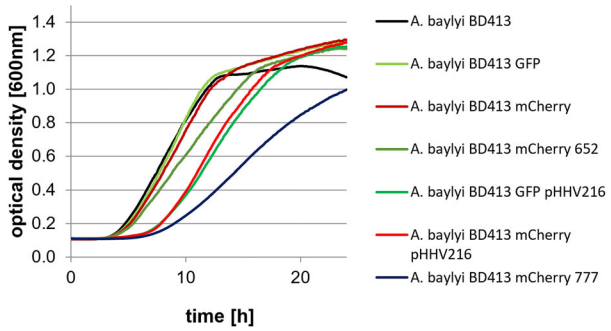

FIGURE 1 Growth curves of labelled strains and selected mutants in Müller Hinton medium (MH). The figure represents a typical graph of three independent experiments.

TABLE 2 The mutations of *A. baylyi* BD413 mCherry 652 and *A. baylyi* BD413 mCherry 777

Position	Mutation	Effect
***A. baylyi* BD413 mCherry 652**		
1,356,416-1,364,972	deletion of 8556 bp	Deletion of nine genes
***A. baylyi* BD413 mCherry 777**		
713,010	deletion of 2 bp	Truncation of *bfms*
1,362,849	A → G	L212S AdeN homologue (ACIAD1367)
2,531,010	G → A	Silent mutation
3,357,842	G → A	Silent mutation

of MICs and MSCs showed, that for most antibiotics the MSCs were 2-fold to 10 fold lower than the MICs of the susceptible strain. Only for the quinolones, the MSCs were in the range of the MICs, and both values were around or below 50 μg/L (Table 3). Low MIC and MSC values were also demonstrated for meropenem (Table 3). Therefore, the concentrations of those antibiotics, which define the most problematic resistance phenotypes (resistance to meropenem, ciprofloxacin and piperacillin) were determined in hospital wastewater.

Antibiotic residues in hospital wastewater

Because of the large fluctuation range in antibiotic concentrations during earlier measurements (Voigt *et al*. 2020b), the monitoring was performed in a continuous manner, i.e., 1 h mixed samples were taken at hourly intervals from the main wastewater sewer of the hospital on eleven different days (Figure 3). The analysis revealed marked antibiotic concentration peaks at 6:15 a.m. and 8:15 p.m. as well as a lower peak at 12:15 pm. These concentration peaks with single outliers in the mg/L range were very distinct for meropenem and piperacillin and less pronounced for ciprofloxacin. The meropenem MSCs of the two mutant strains (30–41 μg/mL) of *A. baylyi* were always lower than the median concentrations, which surpassed 100 μg/L in nearly all samples. Meropenem concentrations peaks (up to 1900 μg/L) were much higher than the MIC of the susceptible isolate (MIC <125 μg/L) and even surpassed the MICs of one mutant. For piperacillin, which reached the highest concentrations of all antibiotics measured (up to >3500 μg/L), the MSCs of our mutants (1622 μg/mL) were only met by the concentrations in the wastewater during peak times. For ciprofloxacin, the lowest MSC (17 μg/L) was within the concentration range measured for most of the time and the MICs were exceeded in the samples with the highest concentrations.

DISCUSSION

The concentrations of antibiotics that are released into the environment vary greatly depending on source and treatment of wastewater. Extremely high concentrations, that exceed the MICs of susceptible bacteria, have been measured in the wastewater of facilities that produce antibiotics (e.g., 1,000,000 μg/L oxytetracycline decreasing to 80,000 μg/L after first purification steps) (Li *et al*. 2008). High concentrations are also present in the drains of sinks, showers, and toilets of hospital wards with patients that receive antibiotic therapy (Voigt *et al*. 2019) as well as in raw hospital wastewater sampled at the outlet into the main sewer (Voigt *et al*. 2020b). The continuous measurements here showed that the meropenem and piperacillin concentrations in hospital wastewater varied over a great range with maximum values of up to 3500 μg/L. Most probably, these fluctuations reflected the daily rhythm of the hospital patients as well as the work schedule on the wards, including the emptying of urine bags at the end of shifts. The fluctuations were not so distinct during the day between 8 a.m. and 5 p.m., when the wastewater from the wards was diluted with wastewater from the administrative and educational departments as well as scientific institutes located on the same campus. Similar fluctuations of quinolone concentrations in hospital wastewater have been recently reported (Cai *et al*. 2022). High concentrations above the MIC of *A. baylyi* BD413, as measured here for the carbapenem meropenem, will promote the growth and survival of carbapenem resistant bacteria, which belong to the critical priority pathogens listed by the WHO (WHO 2017). These bacteria may cause nosocomial infections that are difficult to treat. Therefore, their dissemination is a major concern and should be avoided (Bonomo *et al*. 2018). Intermittent antibiotic exposure, i.e., concentrations that fluctuate above and below the MIC, select additionally for tolerance mutations (Levin-Reisman *et al*. 2017). Tolerance rescues bacteria from

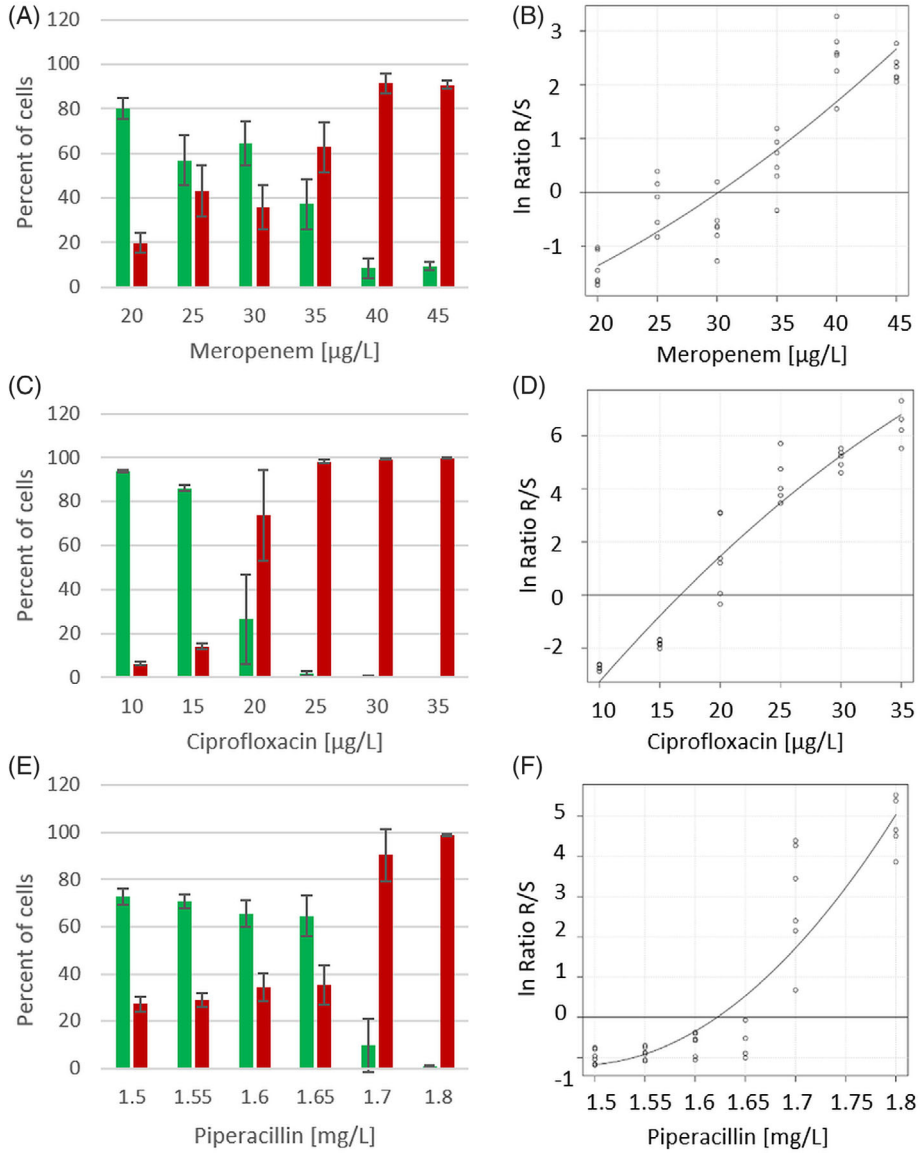

FIGURE 2 MSC determinations. Mean cell counts (percent) of six independent MSC experiments using the strains *A. baylyi* BD413 GFP (green bars) and *A. baylyi* BD413 mCherry 777 (red bars) and three different antibiotics, A) meropenem, C) ciprofloxacin, and E) piperacillin, after 24 h of incubation. Whiskers indicate the standard deviations. To obtain the MSC, the natural logarithms of the ratio resistant versus susceptible cells were plotted against the antibiotic concentrations and are shown in B) for meropenem, D) for ciprofloxacin and F) for piperacillin. The MSC is the intersection of the x-axis with the regression curve.

bactericidal antibiotics that require growth for their action, like quinolones and β-lactams (ciprofloxacin, meropenem and piperacillin), without changing their MICs (Fridman *et al*. 2014). However, tolerant bacteria tend to develop resistance mutations and, therefore, selection of tolerant bacteria also presents a certain risk (Levin-Reisman *et al*. 2017; Fridman *et al*. 2014). The peak concentrations of ciprofloxacin in hospital wastewater were in the range or above the MSCs of the *A. baylyi* test organisms and, therefore, favored the growth of strains with decreased susceptibility. Considering that carbapenems and ciprofloxacin reached critical levels in the range of the MICs or MSCs in the hospital wastewater, the presence of diverse bacteria that were simultaneously resistant to ciprofloxacin and carbapenems is not surprising (Müller *et al*. 2018; Kehl *et al*. 2022; Sib *et al*. 2020). Such high concentrations that are in the range of MICs and MSCs also explain the difficulties that are encountered, when elimination of these organisms from hospital drains is attempted (Kizny Gordon *et al*. 2017). The fact that meropenem probably exerts the strongest effect was unexpected, since β-lactams are usually short-lived in wastewater (Ribeiro *et al*. 2018). However, in hospital wastewater a constant emission of these antibiotics is likely to maintain high concentrations.

At the influent into the wastewater plant, the antibiotic concentrations in hospital wastewater are generally lower, however, even here the maximum values of quinolones, penicillins and tetracyclines may still be in the

TABLE 3 MSC values of the strains *A. baylyi* BD413 GFP versus *A. baylyi* BD413 mCherry pHHV216 or *A. baylyi* BD413 mCherry versus *A. baylyi* BD413 GFP pHHV216 (both combinations with pHHV216 are represented by "pHHV216"), *A. baylyi* BD413 GFP versus *A. baylyi* BD413 mCherry 652, and *A. baylyi* BD413 GFP versus *A. baylyi* BD413 mCherry 777.

Antibiotic	Resistant strain	MSC [μg/L]	2.5th percentile	97.5th percentile	R^2	MIC*	MSC/MIC**
Chloramphenicol	pHHV216	748.36	603.1	898.5	0.84	4000.0	0.19
Tetracycline	pHHV216	53.65	41.1	68.8	0.90	500.0	0.11
Sulfamethoxazole	pHHV216	297.90	269.7	324.7	0.90	3000.0	0.10
Piperacillin	mCherry 652	1622.47	1589.8	1654.1	0.85	4000.0	0.41
	mCherry 777	1668.53	1647.1	1688.6	0.83		0.42
Levofloxacin	mCherry 652	43.17	25.0	58.7	0.94	39.0	1.11
	mCherry 777	46.98	37.0	56.7	0.99		1.20
Ciprofloxacin	mCherry 652	16.74	15.7	17.8	0.94	37.0	0.45
	mCherry 777	51.51	51.2	51.8	0.97		1.39
Meropenem	mCherry 652	41.08	40.0	42.0	0.92	125	0.33
	mCherry 777	30.14	27.9	32.2	0.82		0.24
Clindamycin	mCherry 652	687.83	492.6	938.5	0.84	8000.0	0.09
	mCherry 777	1000.22	851.1	1180.5	0.93		0.13

*MIC of the susceptible strain in MSC determination.
**MSC resistant mutant / MIC susceptible strain.

range of the MSCs measured here (Novo *et al*. 2013; Verlicchi *et al*. 2012). Verlichi et al (2012) sampled raw hospital effluents and found a peak concentration of 26 μg/L ciprofloxacin. In another study with raw wastewater influent and a mixed bacterial community, there was a positive selection for *intI*1 between 7.8 and 15.6 μg/L ciprofloxacin, which aligns well with the values measured here (Stanton *et al*. 2020). In contrast, antibiotic concentrations in the treated effluents of wastewater plants are normally in the ng/L range (for a review please see (Michael *et al*. 2013)), or reach low μg/L values, e.g., 1.5 μg/L sulfamethoxazole (Sinthuchai *et al*. 2016) or 1.4 μg/L (peak value) ciprofloxacin (Rodriguez-Mozaz *et al*. 2020) and thus are lower than the MSCs of *A. baylyi*. But even in the presence of 1 μg/L tetracycline, *tetA* and *tetG* accumulated in biofilms (Lundström *et al*. 2016), *qnr* genes could be selected with 1 μg/L ciprofloxacin, and resistant *E. coli* harboring *gyrA* mutations increased in a mixed community at 5–10 μg/L (Kraupner *et al*. 2018). These values are even slightly lower than the lowest MSC measured here. In another example, cefotaxime exposure enriched $bla_{CTX\text{-}M}$ even at an MSC of 0.4 μg/L (Murray *et al*. 2018). In conclusion, raw wastewater may contain selective concentrations of antibiotics and even after partial removal of antibiotics by wastewater treatment, effects on selection of resistance genes are possible. In addition, hospital wastewater represents a complex mixture of different substances (Lindberg *et al*. 2014; Verlicchi *et al*. 2012; Voigt *et al*. 2019) and there is only little knowledge of the effect of these mixtures on bacteria. Only an enrichment of the carbapenemase OXA-48 has been described (Bengtsson-Palme *et al*. 2016).

The spontaneous mutants (Table 2) selected on cephalosporin containing agar in this study showed a decreased susceptibility to various antibiotics. Genomic sequencing showed a number of point mutations concerning regulatory genes in strain *A. baylyi* BD413 mCherry 777, one of this being a truncation of the sensor kinase BmfS by a frameshift. In *A. baumannii*, BmfS is involved in biofilm production, i.e. the regulation of pilin biosynthesis, motility, and quorum sensing (Tomaras *et al*. 2008), but also antibiotic resistance, since inactivation of *bfmS* in *A. baumannii* yielded a decrease in susceptibility against ciprofloxacin (Liou *et al*. 2014). Both strains harbored mutations in AdeN which represses the efflux pump AdeIJK (ACIAD2943-ACIAD2945) in *A. baumannii* (Rosenfeld *et al*. 2012). An unspecific effect of upregulation of AdeIJK on the susceptibility against multiple antibiotics and biocides in *Acinetobacter* has been reported before and might be present in both isolates. In *A. baylyi* this affected susceptibility to chloramphenicol, ethidium bromide, benzalkonium chloride, norfloxacin, tetracycline, and trimethoprim (Brzoska *et al*. 2013) and in *A. baumannii* susceptibility to moxifloxacin, cefepime, meropenem, ertapenem, macrolides, ciprofloxacin, tigecycline, and trimethoprim/sulfamethoxazole was decreased (Fernando *et al*. 2014). In *A. baylyi* BD413 mCherry 777, this repressor harbors an exchange (L212S AdeN). Although it is difficult to estimate the effect of this exchange, the original leucine residue is conserved in all sequences of this protein that are stored in the database NCBI. The promoter of the intrinsic $bla_{ADC\text{-}8}$ gene was unchanged. The strain itself was characterized by decreased susceptibility to quinolones, sulfamethoxazole/trimethoprim, clindamycin, erythromycin, and tetracycline and resistance to nearly all tested β-lactams, a prolonged lag-phase in growth experiments and slow growth. In the second

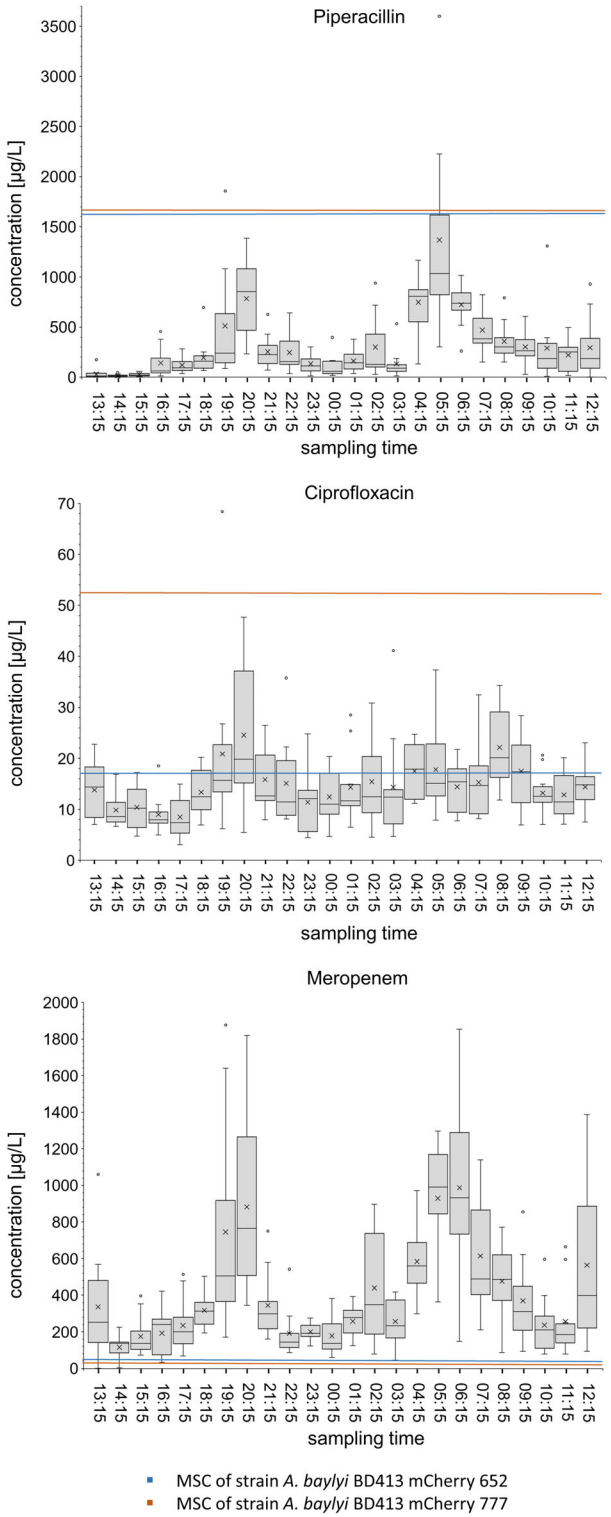

FIGURE 3 Diurnal antibiotic concentrations measured in raw clinical wastewater. Each box represents the concentrations present in 11 different hourly composite samples, which, in turn, comprise a mixture of 30 subsamples taken every 2 minutes. Mean values are indicated by (x), median values are indicated by the bar and whiskers limit the range of values that are not considered outliers. Outliers (o) are defined as values that exceed the interquartile range 1.5 times. The MSCs of strain *A. baylyi* BD413 mCherry 652 (blue) and *A. baylyi* BD413 mCherry 777 (orange) have been marked.

isolate, *A. baylyi* BD413 mCherry 652, *adeN* had been deleted causing only low fitness costs, which may have be the cause for the low MSC for ciprofloxacin in this strain. There have been reports, that a strong activity of the efflux pump AdeIJK is toxic in *A. baumannii* (Damier-Piolle *et al*. 2007), however, this did not seem to be the case in *A. baylyi*. The MICs of quinolones, piperacillin, ceftazidime, and meropenem were increased in this isolate and may have been influenced by an upregulation of AdeIJK. *A. baylyi* BD413 mCherry 652 reached EUCAST breakpoint levels of resistance only for ceftazidime and ceftazidime/avibactam, i.e., for the other antibiotics the benefit of the mutations was small. Simultaneously, the fitness costs of the mutations were lower than those of *A. baylyi* BD413 mCherry 777 or the plasmid-carrying variants. The combination of low MICs and low fitness costs resulted in very low MSCs, which might be misleading if considered for risk assessments. For ciprofloxacin, our parent and mutant strains fall into the category "susceptible, increased exposure" and MICs and MSCs are low. However, very low MSCs have also been described for ciprofloxacin resistance mediated by chromosomal mutations in *gyrA* and *parC*. For example, concentrations as low as 5–10 µg/L selected resistant strains, due to the low fitness costs of these mutations in aquatic communities (Kraupner *et al*. 2018) and even lower concentrations (0.1 µg/mL) selected such mutants in pure culture competition experiments (Gullberg et al., 2011). Considering that many problematic clones in the hospital (ST235 *Pseudomonas aeruginosa* (Treepong *et al*. 2018), ST147 *Klebsiella pneumoniae* (Pitout *et al*. 2019)) have been selected by usage of ciprofloxacin after acquiring *parC* and *gyrA* mutations, the concentrations of quinolones should be closely monitored.

In conclusion, we have shown here that growth of variants of *A. baylyi* with decreased susceptibility would be selected by antibiotic concentrations that are present in raw hospital wastewater. The antibiotic concentration measurements and MSCs indicate that high antibiotic concentrations, especially of ciprofloxacin and concentration peaks of β-lactams, may be the drivers in the selection of multi-resistant bacteria in this biotope (Sib *et al*. 2020; Kehl *et al*. 2022) and must be taken into account, when remediation measures are planned.

Experimental Procedures

Bacterial strains and plasmids

Table S1 lists strains and plasmids. In order to label *A. baylyi* BD413 with fluorescent proteins, it was transformed with the gene cassette presented in **Figure S1**, containing a strong P5 bacteriophage promoter derived

from the plasmid pEPSA5 (Forsyth et al., 2002), encoding either GFP (GenBank Acc. no. AAA27721.1) or mCherry (GenBank Acc. no. PWI63952.1) and a resistance-cassette for spectinomycin (APH[3′]-IIIa) derived from *Staphylococcus aureus* serving as selection marker (GenBank accession no. MVJ63870.1). The nucleotide sequences of the gene cassette (1752 bp for the *gfp* version and 1746 bp for the *mCherry* version) were adapted to the codon usage of *Acinetobacter* and were synthesized *de novo* by BaseClear B.V. (Leiden, Netherlands) with terminal BamHI and KpnI restriction sites. The cassette was amplified using BKon_for and Spec_rev2 (**Table S2**). After that, both ends of the cassette were digested and ligated to two different fragments of the gene *lipA* of *A. baylyi* BD413 amplified with the primer combinations LipA1_for/LipA1_rev2 and LipA2_for3/LipA2_rev (**Table S2**). The ligation products were amplified by PCR using LipA1_for and LipA2_Rev. All PCR reactions were performed on a Thermocycler from Sensoquest GmbH (Göttingen, Germany). *A. baylyi* BD413 was transformed with the resulting PCR products using competence transformation (see below) and the suspension was plated on LB agar containing spectinomycin (150 μg/mL). Successful transformants (*A. baylyi* BD413 GFP and *A. baylyi* BD413 mCherry) were detected by fluorescence. The correct integration of the cassette was confirmed by PCR and sequencing.

Both strains were transformed with the low GC-plasmid pHHV216 (Heuer *et al*. 2009). Successful transformants were selected on agar plates containing sulfadiazine (100 mg/L), streptomycin (20 mg/L), spectinomycin (150 mg/L) and tetracycline (5 mg/L). In conclusion, the procedure yielded two differently labelled strain pairs (*A. baylyi* BD413 GFP (susceptible) versus *A. baylyi* BD413 mCherry pHHV216 (resistant) and *A. baylyi* BD413 GFP pHHV216 (resistant) versus *A. baylyi* BD413 mCherry (susceptible)) for competition experiments.

Spontaneous mutants

Spontaneous resistant mutants of *A. baylyi* BD413 mCherry were selected on Chromagar™ ESBL plates (Mast Diagnostica GmbH, Reinfeld, Germany). After plating, selective agar plates were incubated at 28°C for 24 h or until colonies became visible.

Competence transformation

Plasmid DNA was prepared by heating 100 μL of an overnight culture of the donor cells for 5 min to 100°C. After that, the cells were spun down and the supernatant was used as DNA preparation. PCR products were purified using the GeneJet™ PCR Purification Kit (Thermo Fisher SCIENTIFIC, Dreieich, Germany). For competence transformation, the method of Palmen et al. (1993) was modified slightly (Palmen *et al*. 1993). *A. baylyi* BD413 or its labelled variants were shaken at 180 rpm in 5 mL Luria Bertani (LB) broth in a glass tube at 30°C overnight. One mL of this pre-culture was then diluted with 25 mL of fresh LB in a 100 ml Erlenmeyer flask and shaken further for 2 h. The transformation was initiated by mixing 0.5 mL of this culture with 2 μg of DNA in a fresh glass tube. The tube was shaken for another 4–6 hours. For selection of transformed colonies, the bacteria were plated onto agar plates with the corresponding antibiotics.

Sequencing

Genomic DNA was prepared from 1–3 ml of culture employing the MasterPure™ Complete DNA and RNA Purification Kit from Lucigen (Middleton, USA) according to the manufacturer's instructions. DNA concentration and purity was determined using a NanoDrop One^{C} spectrophotometer (Thermo Fisher SCIENTIFIC, Dreieich, Germany) and the Invitrogen Qubit™ fluorometer with the Qubit™ 1x dsDNA HS Assay Kit (Thermo Fisher SCIENTIFIC, Dreieich, Germany). For *A. baylyi* BD413 mCherry 777 and *A. baylyi* BD413 mCherry 652 the library was prepared employing the Illumina© Nextera XT DNA Library Preparation Kit and the Illumina© Nextera XT Index Kit (Illumina, San Diego, USA). For sequencing, the Illumina V3 kit (600 cycles, 2 x 300 bp) was used on a MiSeq Sequencing System. The reads were assembled *de novo* using the Geneious assembler (version 10.0) and compared to the genome of *A. baylyi* BD413 using the Mauve plugin. For the detection of point mutations, raw reads were mapped to the genome sequence of *A. baylyi* BD 413 (NC_005966.1) using the Geneious read mapper (version 10.0) (http://www.geneious.com).

Amino acid exchanges and the deletion in *A. baylyi* BD413 variants were confirmed by PCR and Sanger sequencing using primers listed in **Table S2**. Sequencing of PCR products was performed by Microsynth (Balgach, Switzerland) or Eurofins Genomics (Ebersberg, Germany).

Antibiotic susceptibility testing

The susceptibility against different antibiotics was tested employing the Micronaut-MDR MRGN Screening 3 system (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-Hersel, Germany) and interpreted according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2022). Manual MIC determinations followed the guidelines for broth micro-dilution method of the

Clinical and Laboratory Standards Institute (CLSI) in 96-well polystyrene round-bottomed microplates (Greiner Bio-One International GmbH, Kremsmünster, Austria) in Mueller-Hinton Broth (MH, Oxoid Limited, Basingstoke, United Kingdom) using an inoculum of 3.5 x 10^5 cells per mL. The microtiter plates were incubated at 30°C and 180 rpm for 24 hours in a shaker (Infors HT Ecotron, Bottmingen-Basel, Switzerland).

Minimal selective concentration (MSC)

For the competition experiments, antibiotics that reached high concentrations in raw clinical wastewater (ciprofloxacin, meropenem, piperacillin), that are persistent in the environment (sulfamethoxazole, quinolones), that are covered by resistance genes on the plasmid pHHV266 (chloramphenicol, tetracycline) or that showed a lower activity against the variants (clindamycin) were chosen. Precultures in MH broth of both competing strain pairs were shaken in separate tubes at 30°C and 180 rpm on a horizontal shaker overnight. Both strain pairs (*A. baylyi* BD413 GFP versus *A. baylyi* BD413 mCherry pHHV216 and *A. baylyi* BD413 GFP pHHV216 versus *A. baylyi* BD413 mCherry) were always tested in parallel. On the next day, the cultures were adjusted to an optical density (OD_{600nm}) of 1.0 using fresh MH broth and then diluted 1:1000 with MH. For the competition experiments, a number of test tubes containing a variety of different antibiotic concentrations in 5 ml MH medium had been prepared beforehand. At the start of the experiment, the test tubes were inoculated with 10 μL of the above dilutions with each strain. The incubation took place at 37°C, 170 rpm in an orbital shaker for 20 h. The results of the competition assays were evaluated either by direct plating of diluted cultures on agar plates (without any antibiotics) or by fluorescence activated cell sorting (FACS) after fixation of the culture. To this end, the whole culture was centrifuged in 2 mL Eppendorf tubes for 2 min at 16000 *g* and washed twice with 1 mL sterile 10% glycerol. This was followed by a washing step with 1 mL autoclaved, deionized water and the addition of 100 μL fixation buffer (BioLegend, San Diego, CA, USA). The tubes were then incubated in the dark for 60 min at room temperature. In preparation for the measurement, the samples were diluted 1:200 with sterile, deionized water to a total volume of 2 m. The bacteria were counted using a Cytoflex S flow cytometer employing the CytExpert Software version 1.2 (Beckman Coulter, Brea, USA) or with a BD LSRFortessaTM Cell Analyzer with the software BD FACSDivaTM version 8.0.1 (BD Biosciences, New York, USA). In control experiments, aliquots of cultures from the MSC determinations with the plasmid bearing strains were plated onto MH agar plates containing sulfadiazine (100 mg/L), streptomycin (20 mg/L), and tetracycline (5 mg/L). After an incubation of 24 h, the plates were checked for growth of the susceptible plasmid-free strain using a UV lamp (Chroma 43, Vetter GmbH, Wiesloch, Germany) in order to exclude plasmid transfer to the susceptible strain. Normally a first orientation experiment contained the antibiotic in 1:10 dilution steps covering a wide range of concentrations below the MIC. A second determination comprising six independent measurements was then performed with concentrations that were close to the MSC.

MSC data analysis

The MSC is defined as the antibiotic concentration at which the growth of susceptible bacteria is equal to the growth of resistant bacteria (Greenfield *et al*. 2018). Hence, the ratio between resistant and susceptible bacteria must be equal to one at the MSC. Therefore, ratios (resistant cells/susceptible cells) for different antibiotic concentrations of all measurements were logarithmized using the natural logarithm and fitted to first- and second-order polynomials, employing the linear least-square algorithm. The MSC was determined as the intersection of the polynomial with the x-axis. A bootstrapping algorithm was used to determine the uncertainty of the MSC (Efron 1979). Bootstrapping is described as a smoothed version of cross-variance (Efron and Tibshirani 1997). In this method, the collected datapoints are resampled with replacement to create a large number of bootstrapping samples. The 95% confidence interval was defined as the interval between the 2.5th percentile and the 97.5th percentile of the determined MSCs of the bootstrap samples. All calculations were done in R (R Core Team 2022). R^2 values using the second order polynomial were always larger than the values obtained using the first order curves and according to ANOVA both models differed significantly. Therefore, the values obtained using the second order polynomial were selected for Table 3 and results obtained with both models as well as p-values are listed in Table S3.

Sequencing data analysis

Unless stated otherwise, sequence data was processed with Geneious version 10 (Biomatters Ltd., Auckland, New Zealand). The Mauve plug in Geneious was used for comparative alignments (Darling *et al*. 2010).

Determination of growth rates

For the characterization of growth rates, the different strains were grown overnight in MH medium. These

precultures were adjusted to an OD_{600} of 1.0 and thereafter diluted 1:1000. One hundred µl of this suspension, containing about 7 x 10^5 cells per ml, served as inoculum for each well in a 96-well polystyrene round-bottomed microplate (Greiner Bio-One International GmbH) filled with 100 µl of MH. The plate was incubated at 30°C for 24 h with shaking for 500 s in between the measurements of the optical density at 600 nm (OD_{600nm}) using a Tecan Infinite® 200PRO (Tecan Trading AG, Männedorf, Switzerland) with the software Tecan i-control version 2.0.10.0.

Measurements of antibiotic concentrations

Hospital wastewater was obtained from the main sewer of a maximum care hospital with a high case severity (over 1200 beds and 8300 employees), which collects wastewater from clinical units, administrative buildings and associated research institutes amounting to 27.1 m^3/h (Voigt *et al*. 2020b). The sewer was sampled at hourly intervals on eleven different working days. Samples were taken using a BÜHLER 2000 automatic sampler (HACH LANGE, Düsseldorf, Germany). For every hour, one composite sample was obtained. This sample consisted of 30 individual samples taken every 2 minutes with a volume of 25 ml each. The samples were filled into glass vessels and cooled to 4°C in the sample room of the BÜHLER 2000 before transport to the laboratory. Antibiotic concentrations of ciprofloxacin, meropenem, and piperacillin were determined as previously described (Voigt *et al*. 2020a; Voigt *et al*. 2020b). Levofloxacin concentrations were not measured although showing an effect, because its usage in the clinics is very low and it cannot be distinguished from the racemate ofloxacin.

Accession numbers

The nucleotide sequences of *A. baylyi* BD413 (Barbe *et al*. 2004; EUCAST 2022) and the plasmid pHVV216 are available from NCBI under acc. no. NC_005966.1 and acc. no. FJ012880.1. Sequencing reads were submitted to ENA (acc. no.PRJEB50487).

ACKNOWLEDGEMENTS

We would like to acknowledge the assistance of the Flow Cytometry Core Facility at the Institute of Experimental Immunology, Medical Faculty at the University of Bonn. In addition, we want to thank M. Ritter at the Institute of Medical Microbiology, Immunology and Parasitology, Medical Faculty at the University of Bonn for his kind support with the FACS measurements and P. Stevens, U. Neder and S. Unsleber at Noscendo GmbH for help with sequencing. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

The study was funded by a grant of the BMBF (Federal Ministry of Education and Research of Germany) as a part of the collaborative research project "HyReKA" [grant number FKZ 02WRS1377 to GB, HF and ME] and by the Deutsche Forschungsgemeinschaft (For 5095, DFG, German Research Foundation to GB, KS, and CZ, project number 431531292).

CONFLICT OF INTEREST

None declared.

REFERENCES

Al Atrouni, A., Joly-Guillou, M.-L., Hamze, M. & Kempf, M. (2016) Reservoirs of non-*baumannii Acinetobacter* species. *Frontiers in Microbiology*, 7, 49.

Andersson, D.I. & Hughes, D. (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews. Microbiology*, 8, 260–271.

Barbe, V., Vallenet, D., Fonknechten, N., Kreimeyer, A., Oztas, S., Labarre, L. et al. (2004) Unique features revealed by the genome sequence of *Acinetobacter* sp. ADP1, a versatile and naturally transformation competent bacterium. *Nucleic Acids Research*, 32, 5766–5779.

Beceiro, A., Pérez-Llarena, F.J., Pérez, A., Del Tomás, M.M., Fernández, A., Mallo, S. et al. (2007) Molecular characterization of the gene encoding a new AmpC beta-lactamase in *Acinetobacter baylyi*. *The Journal of Antimicrobial Chemotherapy*, 59, 996–1000.

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C.-F. et al. (2016) Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci Total Environ*, 572, 697–712.

Bonomo, R.A., Burd, E.M., Conly, J., Limbago, B.M., Poirel, L., Segre, J.A. et al. (2018) Carbapenemase-producing organisms: A global scourge. *Clinical Infectious Diseases*, 66, 1290–1297.

Broszat, M., Nacke, H., Blasi, R., Siebe, C., Huebner, J., Daniel, R. et al. (2014) Wastewater irrigation increases the abundance of potentially harmful gammaproteobacteria in soils in Mezquital Valley, Mexico. *Applied and Environmental Microbiology*, 80, 5282–5291.

Brzoska, A.J., Hassan, K.A., Leon, E.J. de, Paulsen, I.T., and Lewis, P. J. (2013) Single-step selection of drug resistant *Acinetobacter baylyi* ADP1 mutants reveals a functional redundancy in the recruitment of multidrug efflux systems. *PLoS One*, 8: e56090.

Cai, M., Wang, Z., Gu, H., Dong, H., Zhang, X., Cui, N. et al. (2022) Occurrence and temporal variation of antibiotics and antibiotic resistance genes in hospital inpatient department wastewater: Impacts of daily schedule of inpatients and wastewater treatment process. *Chemosphere*, 292, 133405.

Carr, E.L., Kämpfer, P., Patel, B.K.C., Gürtler, V. & Seviour, R.J. (2003) Seven novel species of *Acinetobacter* isolated from activated sludge. *International Journal of Systematic and Evolutionary Microbiology*, 53, 953–963.

Damier-Piolle, L., Magnet, S., Brémont, S., Lambert, T. & Courvalin, P. (2007) AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy*, 52, 557–562.

Darling, A.E., Mau, B. & Perna, N.T. (2010) progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One*, 5, e11147.

Efron, B. (1979) Bootstrap methods: Another look at the Jackknife. *The Annals of Statistics*, 1, 1–26.

Efron, B. & Tibshirani, R. (1997) Improvements on cross-validation: the 632+ bootstrap method. *Journal of the American Statistical Association*, 92, 548–560.

Elliott, K.T. & Neidle, E.L. (2011) *Acinetobacter baylyi* ADP1: transforming the choice of model organism. *IUBMB Life*, 63, 1075–1080.

EUCAST (2022) European committee on antimicrobial susceptibility testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0, valid from 2022-01-01. [WWW document]. URL http://www.eucast.org.

European Centre for Disease Prevention and Control (2020) Antimicrobial consumption in the EU/EEA – Annual Epidemiological Report 2019. [WWW document]. URL https://www.ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-consumption-europe-2019.

Fernando, D.M., Xu, W., Loewen, P.C., Zhanel, G.G. & Kumar, A. (2014) Triclosan can select for an AdeIJK-overexpressing mutant of *Acinetobacter baumannii* ATCC 17978 that displays reduced susceptibility to multiple antibiotics. *Antimicrobial Agents and Chemotherapy*, 58, 6424–6431.

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N. & Balaban, N.Q. (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature*, 513, 418–421.

Greenfield, B.K., Shaked, S., Marrs, C.F., Nelson, P., Raxter, I., Xi, C. et al. (2018) Modeling the emergence of antibiotic resistance in the environment: an analytical solution for the minimum selection concentration. *Antimicrobial Agents and Chemotherapy*, 62, e01686–e01617.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D. et al. (2011) Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathogens*, 7, e1002158.

Heuer, H., Kopmann, C., Binh, C.T.T., Top, E.M. & Smalla, K. (2009) Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low % G+C content. *Env Microbiol*, 11, 937–949.

Hughes, D. & Andersson, D.I. (2012) Selection of resistance at lethal and non-lethal antibiotic concentrations. *Current Opinion in Microbiology*, 15, 555–560.

Jechalke, S., Kopmann, C., Richter, M., Moenickes, S., Heuer, H. & Smalla, K. (2013) Plasmid-mediated fitness advantage of *Acinetobacter baylyi* in sulfadiazine-polluted soil. *FEMS Microbiology Letters*, 348, 127–132.

Juni, E. & Janik, A. (1969) Transformation of *Acinetobacter calcoaceticus* (*Bacterium anitratum*). *Journal of Bacteriology*, 98, 281–288.

Jutkina, J., Rutgersson, C., Flach, C.-F. & Joakim Larsson, D.G. (2016) An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. *Sci Total Environ*, 548-549, 131–138.

Karah, N., Jolley, K.A., Hall, R.M. & Uhlin, B.E. (2017) Database for the *ampC* alleles in *Acinetobacter baumannii*. *PLoS One*, 12, e0176695.

Kehl, K., Schallenberg, A., Szekat, C., Albert, C., Sib, E., Exner, M. et al. (2022) Dissemination of carbapenem resistant bacteria from hospital wastewater into the environment. *Sci Total Environ*, 806, 151339.

Khan, S., Beattie, T.K. & Knapp, C.W. (2017) The use of minimum selectable concentrations (MSCs) for determining the selection of antimicrobial resistant bacteria. *Ecotoxicology*, 26, 283–292.

Kizny Gordon, A.E., Mathers, A.J., Cheong, E.Y.L., Gottlieb, T., Kotay, S., Walker, A.S. et al. (2017) The hospital water environment as a reservoir for carbapenem-resistant organisms causing hospital-acquired infections - a systematic review of the literature. *Clinical Infectious Diseases*, 64, 1435–1444.

Klümper, U., Recker, M., Zhang, L., Yin, X., Zhang, T., Buckling, A. et al. (2019) Selection for antimicrobial resistance is reduced when embedded in a natural microbial community. *The ISME Journal*, 13, 2927–2937.

Kraupner, N., Ebmeyer, S., Bengtsson-Palme, J., Fick, J., Kristiansson, E., Flach, C.-F. et al. (2018) Selective concentration for ciprofloxacin resistance in *Escherichia coli* grown in complex aquatic bacterial biofilms. *Environment Int*, 116, 255–268.

Lesser, L.E., Mora, A., Moreau, C., Mahlknecht, J., Hernández-Antonio, A., Ramírez, A.I. et al. (2018) Survey of 218 organic contaminants in groundwater derived from the world's largest untreated wastewater irrigation system: Mezquital Valley, Mexico. *Chemosphere*, 198, 510–521.

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N. & Balaban, N.Q. (2017) Antibiotic tolerance facilitates the evolution of resistance. *Science*, 355, 826–830.

Li, D., Yang, M., Hu, J., Ren, L., Zhang, Y. & Li, K. (2008) Determination and fate of oxytetracycline and related compounds in oxytetracycline production wastewater and the receiving river. *Environmental Toxicology and Chemistry*, 27, 80–86.

Lin, L., Ringel, P.D., Vettiger, A., Dürr, L. & Basler, M. (2019) DNA Uptake upon T6SS-Dependent Prey Cell Lysis Induces SOS Response and Reduces Fitness of *Acinetobacter baylyi*. *Cell Reports*, 29, 1633–1644.e4.

Lindberg, R.H., Östman, M., Olofsson, U., Grabic, R. & Fick, J. (2014) Occurrence and behaviour of 105 active pharmaceutical ingredients in sewage waters of a municipal sewer collection system. *Water Research*, 58, 221–229.

Liou, M.-L., Soo, P.-C., Ling, S.-R., Kuo, H.-Y., Tang, C.Y. & Chang, K.-C. (2014) The sensor kinase BfmS mediates virulence in *Acinetobacter baumannii*. *Journal of Microbiology, Immunology, and Infection*, 47, 275–281.

Lundström, S.V., Östman, M., Bengtsson-Palme, J., Rutgersson, C., Thoudal, M., Sircar, T. et al. (2016) Minimal selective concentrations of tetracycline in complex aquatic bacterial biofilms. *Sci Total Environ*, 553, 587–595.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T. et al. (2013) Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water Research*, 47, 957–995.

Müller, H., Sib, E., Gajdiss, M., Klanke, U., Lenz-Plet, F., Barabasch, V. et al. (2018) Dissemination of multi-resistant Gram-negative bacteria into German wastewater and surface waters. *FEMS Microbiology Ecology*, 94, fiy057.

Murray, A.K., Zhang, L., Yin, X., Zhang, T., Buckling, A., Snape, J. et al. (2018) Novel insights into selection for antibiotic resistance in complex microbial communities. *mBio*, 9, e00969–e00918.

Novo, A., André, S., Viana, P., Nunes, O.C. & Manaia, C.M. (2013) Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. *Water Research*, 47, 1875–1887.

Östman, M., Lindberg, R.H., Fick, J., Björn, E. & Tysklind, M. (2017) Screening of biocides, metals and antibiotics in Swedish sewage sludge and wastewater. *Water Research*, 115, 318–328.

Palmen, R., Vosman, B., Buijsman, P., Breek, C.K. & Hellingwerf, K.J. (1993) Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*. *Journal of General Microbiology*, 139, 295–305.

Pitout, J.D.D., Peirano, G., Kock, M.M., Strydom, K.-A. & Matsumura, Y. (2019) The global ascendency of OXA-48-Type carbapenemases. *Clinical Microbiology Reviews*, 33, e00102–e00119.

R Core Team. (2022) *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing. [WWW document]. https://www.R-project.org/

Ribeiro, A.R., Sures, B. & Schmidt, T.C. (2018) Cephalosporin antibiotics in the aquatic environment: A critical review of occurrence, fate, ecotoxicity and removal technologies. *Environmental Pollution*, 241, 1153–1166.

Rodriguez-Mozaz, S., Vaz-Moreira, I., Della Varela Giustina, S., Llorca, M., Barceló, D., Schubert, S. et al. (2020) Antibiotic residues in final effluents of European wastewater treatment plants

and their impact on the aquatic environment. *Environment Int*, 140, 105733.

Rohwedder, R., Bergan, T., Thorsteinsson, S.B. & Scholl, H. (1990) Transintestinal elimination of ciprofloxacin. *Chemotherapy*, 36, 77–84.

Rosenfeld, N., Bouchier, C., Courvalin, P. & Périchon, B. (2012) Expression of the resistance-nodulation-cell division pump AdeIJK in *Acinetobacter baumannii* is regulated by AdeN, a TetR-type regulator. *Antimicrobial Agents and Chemotherapy*, 56, 2504–2510.

Sib, E., Lenz-Plet, F., Barabasch, V., Klanke, U., Savin, M., Hembach, N. et al. (2020) Bacteria isolated from hospital, municipal and slaughterhouse wastewaters show characteristic, different resistance profiles. *Sci Total Environ*, 746, 140894.

Sib, E., Voigt, A.M., Wilbring, G., Schreiber, C., Faerber, H.A., Skutlarek, D. et al. (2019) Antibiotic resistant bacteria and resistance genes in biofilms in clinical wastewater networks. *Int J Hygiene Environ Health*, 222, 655–662.

Sinthuchai, D., Boontanon, S.K., Boontanon, N. & Polprasert, C. (2016) Evaluation of removal efficiency of human antibiotics in wastewater treatment plants in Bangkok, Thailand. *Water Science and Technology*, 73, 182–191.

Stanton, I.C., Murray, A.K., Zhang, L., Snape, J. & Gaze, W.H. (2020) Evolution of antibiotic resistance at low antibiotic concentrations including selection below the minimal selective concentration. *Commun Biol*, 3, 467.

Tjandramaga, T.B., Mullie, A., Verbesselt, R., Schepper, P.J.d. & Verbist, L. (1978) Piperacillin: human pharmacokinetics after intravenous and intramuscular administration. *Antimicrobial Agents Chemother*, 14, 829–837.

Tomaras, A.P., Flagler, M.J., Dorsey, C.W., Gaddy, J.A. & Actis, L.A. (2008) Characterization of a two-component regulatory system from *Acinetobacter baumannii* that controls biofilm formation and cellular morphology. *Microbiology*, 154, 3398–3409.

Treepong, P., Kos, V.N., Guyeux, C., Blanc, D.S., Bertrand, X., Valot, B. et al. (2018) Global emergence of the widespread *Pseudomonas aeruginosa* ST235 clone. *Clinical Microbiology and Infection*, 24, 258–266.

Verlicchi, P., Al Aukidy, M., Galletti, A., Petrovic, M. & Barceló, D. (2012) Hospital effluent: investigation of the concentrations and distribution of pharmaceuticals and environmental risk assessment. *Sci Total Environ*, 430, 109–118.

Voigt, A.M., Faerber, H.A., Wilbring, G., Skutlarek, D., Felder, C., Mahn, R. et al. (2019) The occurrence of antimicrobial substances in toilet, sink and shower drainpipes of clinical units: A neglected source of antibiotic residues. *International Journal of Hygiene and Environmental Health*, 222, 455–467.

Voigt, A.M., Skutlarek, D., Timm, C., Schreiber, C., Felder, C., Exner, M. et al. (2020a) Liquid chromatography-tandem mass spectrometry as a fast and simple method for the determination of several antibiotics in different aqueous matrices. *Environment and Chemistry*, 17, 54–75.

Voigt, A.M., Zacharias, N., Timm, C., Wasser, F., Sib, E., Skutlarek, D. et al. (2020b) Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater - An evaluation of clinical influences. *Chemosphere*, 241, 125032.

WHO (2017) Global priority list of antibiotic resistant bacteria to guide research, discovery, and development of new antibiotics. [WWW document]. URL https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1.

Wistrand-Yuen, E., Knopp, M., Hjort, K., Koskiniemi, S., Berg, O.G. & Andersson, D.I. (2018) Evolution of high-level resistance during low-level antibiotic exposure. *Nature Communications*, 9, 1599.

Wolters, B., Hauschild, K., Blau, K., Mulder, I., Heyde, B.J., Sørensen, S.J. et al. (2022) Biosolids for safe land application: does wastewater treatment plant size matters when considering antibiotics, pollutants, microbiome, mobile genetic elements and associated resistance genes? *Environmental Microbiology*, 24, 1573–1589.

Wong, D., Nielsen, T.B., Bonomo, R.A., Pantapalangkoor, P., Luna, B. & Spellberg, B. (2016) Clinical and pathophysiological overview of *Acinetobacter* infections: a century of challenges. *Clinical Microbiology Reviews*, 30, 409–447.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Schuster, D., Axtmann, K., Holstein, N., Felder, C., Voigt, A., Färber, H. et al. (2022) Antibiotic concentrations in raw hospital wastewater surpass minimal selective and minimum inhibitory concentrations of resistant *Acinetobacter baylyi* strains. *Environmental Microbiology*, 24(12), 5721–5733. Available from: https://doi.org/10.1111/1462-2920.16206