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Review

Are the methods used to quantitate mutational and adaptive antibiotic resistance adequate to predict emergence of antibiotic resistance and to identify mechanisms of resistance?

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ABSTRACT

This review discusses the questions if emergence of antibiotic resistance may be affected by the application of different methods for quantification of mutant frequencies and if so far disregarded physiologically relevant conditions at the foci of infection should be considered and if novel analytical methods should be applied. Regulatory authorities are currently requesting information about resistance development following exposure of pathogens either to constant or fluctuating antibiotic concentrations. This optional application of two different methods implies that data should be comparable but they differed by up to eight orders of magnitude when using one method or the other. Mutant-frequencies were low following exposure to constant concentrations but high following exposure to fluctuating concentrations. Furthermore, mutant frequencies differed significantly if bacteria were either routinely grown in commercial media with low mutant frequencies or, as it would be pathophysiologically more relevant, in humanized/ human media with high mutant frequencies. Thus, results generated by using conventional methods may be inconsistent or may even represent artefacts thus causing misinterpretations. Information about modes of action and mechanisms of resistance is requested based on the assumption that target interactions would be identical under different experimental conditions. However, targets being essential under routine conditions get lost under pathophysiologically relevant conditions. Also, the limitation to evaluate cross-resistances within the class only, provided an agent of an existing class is being developed, is just one-sided. Subtle structural homologies between different drug classes and natural compounds are sufficient to select for cross-resistance. Furthermore, cross-resistance emerged due to downstream effects triggered by structurally unrelated antibiotics finally leading into common networks of signal transduction pathways. Therefore, emergence of resistance should not only be addressed from a target-oriented position but also from a physiological perspective linked to human and bacterial physiology, regulatory networks, and from a biochemical perspective considering structural homologies of antibiotics and stressors.

KEYWORDS: mutant-frequencies, constant versus fluctuating concentrations, commercial versus humanized media, conditional expression of targets, networks of resistance signals.

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1. Introduction

The isolation of penicillin G and synthesis of 4'-sulfonamido-2,4-diaminobenzene (codenamed KI 730, prontosil) in 1928 and 1932 are undoubtedly amongst the most beneficial discoveries in human medicine. However, evolution of antimicrobial resistance constitutes a great health threat. It is still widely accepted that antibiotics and antimicrobial resistance act on one hand as weapons providing the the antibiotic-producing organism a competitive advantage in a hostile environment. On the other hand antibiotic resistance protects the antibiotic-producing organism from autotoxic antibiotics. Resistances emerged as a consequence of antibacterial treatment in humans and antibiotic use in agri- or horticulture [1, 2]. Consequently it might well have been assumed that the problem of antibiotic resistance can be overcome by design of next generation drugs with increased antibacterial activities and low propensities for resistance development [3, 4]. However, this assumption proves deceptive. For example, penicillin resistance became a significant problem shortly after its clinical application, such that methicillin-launched in 1960 was followed by identification of methicillin resistance two years later [5]. Vancomycin was launched in 1972 for treatment of methicillin-resistant staphylococci with the firm conviction based on *in vitro* experiments that vancomycin resistance would rather likely not occur. This had long been true until a vancomycin-resistant coagulase-negative staphyloccus was isolated from a diabetes patient in 1986 [6]. Ceftazidime/avibactam (CAZ/AVI) overcomes resistances mediated by Ambler classes A-, C- and some types of D ß-lactamases, and hence it was approved for treatment of carbapenemase-producing *Enterobacteriaceae* and *P. aeruginosa*. Despite low mutant frequencies [7-10] resistance to CAZ/AVI developed sporadically but nevertheless rapidly during treatment of patients with infections due to carbapenemase-producing *Enterobacteriaceae*. The first report of CAZ/AVI resistance emerging and treatment failure was published a few weeks after its approval [11, 12]. Surveillance studies conducted prior to its launch revealed that CAZ/AVI resistance pre-existed at low frequencies [13]. In clinical settings, however, 30% of CAZ/AVI-resistant strains were isolated from treatment-naïve patients [14]. The unexpected presence of pre-existing CAZ/AVI resistance in the treatment-naïve population and fast resistance development associated with clinical failures [14, 15] prompted the European Centre for Disease Prevention and Control (ECDC) to issue a word of caution to the effect that the use of CAZ/AVI had to be handled with care [16]. Clinical development of the leucyl-t-RNA synthase inhibitor epetraborole (GSK2251052/AN3365) has been discontinued because of rapid resistance development in phase II during treatment of complicated urinary tract and intra-abdominal infections although development of *in vitro* resistance was low [17, 18]. Furthermore, it was assumed that resistance against xenobiotics such as fluoroquinolones, i.e. compounds foreign to biological systems, could not develop but the opposite was in fact found to be true. An example is the development of resistance to the 7-substituted fluoroquinolone Y-688 in an experimental MRSA endocarditis model despite its good *in vitro* activity against ciprofloxacin-resistant staphylococci and enterococci and its low propensity for resistance development *in vitro* [19-24], leading to the discontinuation of its development. In addition to this, the development of fluoroquinolone resistance in just one mutated bacterium would require bacterial population densities of at least 10^{27} to 10^{54} CFU/mL assuming that three and even up to six mutation steps each occurring with a mutant frequency of 10^{-9} are necessary to develop clinically relevant levels of resistance [25, 26]; it was therefore thought that fluoroquinolone resistance was unlikely to ever become a problem. However, this emerged rapidly in Gram-negative and Gram-positive species shortly after launch [27-29]. These findings seem to corroborate the Darwinian view regarding the role of antibiotics as selectors of resistance and indirectly as accelerators of innovative drug design. However, antibiotic and even xenobiotic resistance is ancient and evidence will be

presented below that the emergence of resistance is not a monocausal but rather a multifactorial consequence of antibiotic exposure. Furthermore, antibiotic-independent modulations of bacterial physiology and signalling networks cause resistance development. In addition there is the key question why preclinical data and clinical experience differ. Therefore, the parameter "mutant frequency" may possibly neither be predictive nor be clinically relevant.

Bacteria adopt six strategies to sustain the antibiotic "attack": first, mutational events in the bacterial chromosome or accessory DNA; second, recombination of foreign DNA into the bacterial chromosome; third, horizontal transfer of resistance genes; fourth, adaptive processes in response to environmental cues without mutational events; fifth, limited import or augmented export of the antibiotic; and sixth, heteroresistance. Comprehensive reviews [30-41] have summarized the pheno- and genotypes, vertical and horizontal transfer of multiple antibiotic resistances by movable elements, physiological and regulatory mechanisms on which these processes are based as well as the evolutionary, ecological, epidemiological and clinical consequences of antibiotic resistance and environmental selection of antibiotic resistances. Hence these topics will not be addressed once again. This review is limited to discussing the questions whether methodological aspects may have an impact on emergence of mutational or adaptive resistance in preclinical infection models,such that results generated by using conventional methods may be inconsistent or may even represent artefacts thus being misleading, whether the perspective on this topic should possibly be broadened considering so far disregarded triggers stimulating resistance development and.whether novel analytical methods to identify or to predict antibiotic resistances and mechanisms of resistances and cross-resistances, respectively, should be applied.

2. The need for unambiguous terminology

The probability of resistance development is routinely quantitated during antibiotic development. Regulatory authorities request information about the frequency of selection of resistance [42]. Researchers from industry and academia routinely evaluate *in vitro* emergence of resistance by exposing bacteria to constant sub- or supra-inhibitory antibiotic concentrations under static conditions. Alternatively *in vitro* pharmacodynamic models mimicking fluctuating concentrations achieved or predicted in infected patients are used. In the course of single-step experiments either a high- or a relatively low inoculum of bacteria (typically 10^8 to 10^{10} colony forming units (CFU)/mL and about 10^5 CFU/mL, respectively) is exposed to increasing antibiotic concentrations. Following incubation for 18h to 24h the numbers of colonies grown at a given antibiotic concentration are counted and divided either by total number of bacteria inoculated or the number of bacteria grown in the drug-free control at 18h to 24h. Miscellaneous termini such as spontaneous mutation- or mutant-frequency, mutation rate, frequency- or emergence of single step mutation, or single step resistance are used to describe the result thus generated. These different terms used nowadays to describe one single finding were originally used to describe different parameters generated by using various methods. Based on the classical fluctuation test by Luria and Delbrück the mutation rate, μ, describes the relationship between numbers of mutants having acquired a mutation in a single gene against the generation number. The probable number of mutations, m (not the number of mutants), is defined as the number of mutational events per culture [43]. Spontaneous mutations are defined as mutations without a known cause, so that this terminus is a contradiction in itself in the context of drug development as the antibiotic itself is considered to trigger resistance development. The result yielded by using routine procedures to characterize the propensity for resistance development represents rather the mutant frequency which is defined simply as the proportion of mutant bacteria present in a culture following an overnight exposition to the study drug, i.e. the mutant frequency is a discrete endpoint. The mutant frequency is a composite parameter resulting from emergence of new mutations during the incubation period and the number of pre-existing resistant mutants present in the inoculum. The term mutant frequency therefore embraces in itself mutagenicity and selective potential on the one hand and mutability and heterogeneity on the other hand; this being specific for the developmental agent and the specific test strain studied under specific test conditions.

Routine methods used to quantitate antibiotic resistance development thus do not characterize monocausally the probability of *de novo* resistance development. Consequently, it would in principle be interesting to quantitate the two parameters characterizing the antibiotic, i.e. mutagenicity and selective potential, as well as the two parameters describing the biology of the test strain, i.e. mutability and heterogeneity.

3. The need for standardization of methods used

Subtle methodological differences in the investigation of mutant frequencies have a significant impact on the results generated. Some examples only are mentioned pars pro toto in the following. First, the inoculum matters. Ideally, the inoculum should be small to reduce the numbers of pre-existing mutants as much as possible. Therefore, Luria and Delbrück used an inoculum of 50 to 500 CFU/mL in their pioneering studies. However, inocula used nowadays range from 10^5 to 10^{11} CFU/mL, so that the probability of mutants occurring and/or selection of pre-existing resistant subpopulations will be relatively high. For example, minimal inhibitory concentrations (MICs) of fosfomycin for 21 *E. coli* strains with initial values of 1 mg/L each increased 4-, 16- and 256-fold at mean frequencies of 6×10^{-5}, 5.2×10^{-6}, and 5.6×10^{-7}, respectively, in cultures inoculated with 5×10^5 CFU/mL whereas resistant mutants of these strains were no longer detectable if the inoculum was lowered by just 1.5 orders of magnitude. Analogous results were obtained with *K. pneumoniae* [44, 45]. Likewise, *E. coli* mutants resistant to fluoroquinolones emerged in pharmacokinetic/ pharmacodynamic (PK/PD) *in vitro* models more likely in cultures inoculated with 10^7 to 10^9 CFU/mL as compared to an inoculum of 10^5 to 10^6 CFU/mL [46, 47]. It should thus be expected that studies using high inocula currently applied routinely would reveal high mutation rates; however, the opposite is the case. This phenomenon suggests that methods used are probably not selective enough to detect bacteria with a visible antibiotic resistance phenotype.

This assumption suggests that secondly, plating and counting have a significant impact on measurements of mutant frequencies [30, 36]. Experiments are frequently performed just once and – for convenience – only the highest dilutions are plated, so that very small numbers of colonies if any can be counted. Consequently mutant frequencies as low as x-times 10^{-10} to 10^{-11} have been reported [48]. Repetition of experiments at separate times and counting of 50 plates instead of $\leq$10 plates per strain and drug concentration and individual experiment increased numbers of countable colonies significantly, such that mutant frequencies increased by up to six orders of magnitude following exposure of Gram-positive or Gram-negative species to four and two-times their MICs (Table 1) [49]. A larger number of parallel- or repetitive cultures represents the bacterial population much better and counting a higher number of plates gives considerable space to chance as opposed to just a single assay. The comprehensible reduction of work load therefore leads to a decrease in bacterial population diversity and a restriction of statistical coincidence.

Third the so called “jackpot culture” phenomenon first described by Luria and Delbrück has a significant impact on quantifications of mutant frequencies. If a mutational event occurs in the early logarithmic phase, then large numbers of mutant cells will emerge till completion of the experiment [30]. The “jackpot culture” phenomenon indicates that growth phase and incubation period matter too, although the particular time of the mutational event cannot be recorded. Repetitive experiments will minimize the impact of the jackpot culture phenomenon on the end result.

Fourth, antibiotic concentrations matter. Test strains are routinely exposed in single step experiments to multiples of their individual MICs (0.5-, 1-, up to $\geq$16-fold). It is so far not specified if concentration increments are based on a multiplication of the MIC by a factor of 0.5, 2, 4 etc., or on a x-fold increase by $\log_2$ dilution steps with a starting concentration being identical with the individual MIC. A test organism with a MIC of 1 mg/L would be exposed to antibiotic concentrations of 8 mg/L and 256 mg/L, respectively, if the MIC were either be multiplied by a factor of 8 or increased by 8 doubling dilution steps. It is obvious that exposure to very high antibiotic concentrations will not permit any growth at all. In addition, the drug concentrations used should be clinically relevant. Four- and eight-fold increments of MICs

Table 1. Impact of plating and counting on mutant frequencies. Fifty versus ten plates per strain and drug concentration and individual experiment were assessed (modified acc. to 49; bold figures represent the MIC of the individual strain; n.d. = not done).

Species	Moxifloxacin			Ciprofloxacin			Linezolid		
	Concentration	**10 Plates**	**50 plates**	**Concentration**	**10 plates**	**50 plates**	**Concentration**	**10 plates**	**50 plates**
S. aureus	**0.12**	1.1×10^{-7}	1.6×10^{-2}	**1.0**	5.7×10^{-4}	3.6×10^{-2}	**2.0**	4.1×10^{-8}	5.9×10^{-3}
	0.25	6.0×10^{-8}	2.5×10^{-5}	2.0	3.1×10^{-6}	4.2×10^{-5}	4.0	1.2×10^{-9}	2.4×10^{-4}
	0.50	7.1×10^{-8}	4.8×10^{-8}	4.0	1.1×10^{-7}	8.9×10^{-6}	8.0	1.1×10^{-10}	6.2×10^{-7}
S. pneumoniae	**0.06**	1.2×10^{-5}	1.4×10^{-2}	**1.0**	1.0×10^{-6}	1.9×10^{-4}	**1.0**	3.4×10^{-7}	1.9×10^{-1}
	0.12	5.7×10^{-9}	5.7×10^{-9}	2.0	2.6×10^{-6}	5.0×10^{-5}	2.0	2.1×10^{-8}	1.1×10^{-4}
	0.25	1.4×10^{-9}	5.7×10^{-9}	4.0	2.5×10^{-9}	$5{,}7 \times 10^{-7}$	4.0	4.1×10^{-9}	4.1×10^{-9}
E. coli	**0.016**	8.7×10^{-5}	5.6×10^{-2}	**0.008**	4.7×10^{-5}	2.3×10^{-2}	**>8.0**	n.d.	n.d.
	0.032	2.4×10^{-8}	5.6×10^{-5}	0.016	2.4×10^{-8}	4.1×10^{-4}		n.d.	n.d.
	0.064	2.8×10^{-9}	3.2×10^{-7}	0.032	1.4×10^{-9}	1.5×10^{-8}		n.d.	n.d.

are the ones tested most frequently. For example, mutants resistant to ciprofloxacin were generated by exposure of three test strains with MICs of <0.125-, 0.5-, and 1.0 mg/L to either 4-, 8-, and even 16-fold MIC-increments, resulting in drug-exposure concentrations of 0.5-, 2.0-, and 4.0 mg/L, 1.0-, 4.0-, and 8.0 mg/L, and 2.0-, 8.0-, and 16 mg/L, respectively [49-54]. These ciprofloxacin concentrations have to be compared with the mean maximal ciprofloxacin serum concentrations of 1.2- and 2.4 mg/L, respectively, following standard doses of 250- and 500 mg p.o. Mutant frequencies for resistance to ciprofloxacin recorded by using standard single step procedures range from about 10^{-7} to 10^{-10} [46, 51, 54]. Such low mutant frequencies are due to relatively high ciprofloxacin concentrations of 4- to 16-times the MICs whereas exposure of the same strains to clinically relevant lower drug concentrations of one- and twofold the MICs resulted in high ciprofloxacin mutant frequencies ranging from 10^{-1} to 10^{-6} [54]. In general, mutant frequencies increased as drug concentrations decreased independent of) antibiotic class, their modes of action, and bacterial species [48-56] (Table 2). The conduct of multistep exposures of bacteria to $\log_2$-increments of antibiotic concentrations is fraught with an analogous problem. Following overnight incubation bacteria are harvested from the tube with the highest drug concentration permitting growth and are adjusted to appropriate viable counts in order to inoculate a fresh 2-fold dilution series of drug. This process is repeated for 8- to 14 days and longer. MICs are recorded daily and plotted against time to determine if and how rapidly resistance emerged. This multistep procedure implies that bacteria may either be exposed for several days to concentrations even lower than trough concentrations in case almost no increases in MICs could be recorded, or that concentrations increase to supra-inhibitory concentrations exceeding maximal serum concentrations by several-fold. For example, MICs of ciprofloxacin for a methicillin-susceptible *S. aureus* test strain increased from 0.25 to 1,024 mg/L within 7 days [50]. Both, continuous exposures to concentrations lower than trough or significantly higher than maximal concentrations in serum or specialized sites accumulating the drug are clinically irrelevant. Although on the one hand clinically irrelevant drug concentrations are used frequently, on the other hand mutants thus generated provide the possibility of analysing resistance mechanisms and their genetic background.

Fifth, methods used have a significant impact on propensities for resistance development. Regulatory authorities permit the use of either constant or fluctuating concentrations in order to assess the resistance selective and/or mutational potential of the agent. The alternative use of two methods implies that data generated with one method or another should be comparable. However, direct comparisons of static *in vitro* assays and *in vitro* PK/PD models using the same strain and medium revealed that mutant frequencies differed by several orders of magnitude when using one model or another one. Mutant frequencies for resistance to 50 mg/L of fosfomycin differed under routine static conditions in normomutators and hypermutators, being 7.8×10^{-5} and 3.4×10^{-3} respectively. However, mutant frequencies for fosfomycin resistance were $<10^{-1}$ in normo as well as hypermutators in a PK/PD model simulating an i.v. dose of 8g t.i.d. [57]. Colistin exerted a rapid and concentration-dependent *in vitro* bactericidal activity in a static time-kill assay without resistance development whereas colistin resistance emerged rapidly in a PK/PD model [58]. Furthermore, dichotomous resistance development was recorded following colistin treatment of a peritonitis-associated sepsis in mice. Viable counts in peritoneal fluid and spleen of colistin-treated animals amounted to 2.4 and 4.1 $\log_{10}$ CFU/mL as well as 8.1 and 7.2 $\log_{10}$ CFU/mL at 24 hours in animals challenged with 10^7 and 10^8 CFU, respectively, of wild-type *E. coli*. Colistin resistance developed in four of 15 mice challenged with 10^7 CFU but in 15/15 mice challenged with 10^8 CFU whereas *in vitro* mutant frequencies were as low as 3.5×10^{-7}. Resistant clones selected *in vivo* had MICs ranging from 4 to 32 mg/L [59]. Thus, mutant frequencies quantitated in conventional discrete endpoint measurements, PK/PD- or *in vivo*-models, were dependent on the methods applied and results generated with one method or another were not only significantly different but also contradictory. It may be reasonable to assume that the discrepancy between discrete endpoint measurements and PK/PD models could be attributed to an exposure of the test strain either to constant supra-inhibitory concentrations or to fluctuating concentrations

Table 2. Mutant frequencies of various drug classes in Gram-positive and Gram-negative bacteria. (*mean total steady state maximal serum concentrations as specified in the corresponding product information following administration of 500 mg ciprofloxacin p.o., 400 mg moxifloxacin p.o., 400 mg ethylsuccinate erythromycin p.o., 250 mg or 500 mg clarithromycin p.o., 600 mg clindamycin i.v., 600 mg ceftobiprole i.v., 500 mg or 1.000 mg vancomycin i.v., 400 mg p.o., 600 mg p.o. or 600 mg linezolid i.v. (the 600 mg p.o. /i.v. concentrations were used for calculations), 7.5 mg/kg body weight quinopristin/dalfopristin (Q-D; (the quinopristin concentration was used for calculations), 4.0 or 6.0 mg/kg body weight daptomycin, 100 mg minocycline p.o.; 1.000 mg ceftolozane i.v.; 1.000mg ceftazidime i.v.; 500 mg and 1.000 mg imipenem; the shaded areas mark those assays in which drug concentrations to which the corresponding bacterium has been exposed exceeded maximal serum concentrations; in principal, the highest Cmax concentration achieved following administration of the highest dose was used for calculations; **increments of MICs in doubling dilutions; n.d. = not done; it is worth mentioning that total antibiotic concentrations are provided in the product informations whereas the unbound fraction only is antibacterially active).

Agent	Initial MIC (mg/L)	Cmax* (mg/L)	Mutant frequency at				
			1 x MIC	2 x MIC	4 x MIC	8 x MIC	16 x MIC
S. pneumoniae strain No. 1 [49, 50]							
ciprofloxacin	1.0	2.4	$>3.0 \times 10^{-1}$	3.2×10^{-4}	4.6×10^{-7}	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
moxifloxacin	0.125	3.1	1.0×10^{-6}	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$	$<1.0 \times 10^{-10}$
erythromycin	0.06	1.7	1.1×10^{-4}	4.0×10^{-8}	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	n.d.
clarithromycin	0.016	1.5, 3.0	2.5×10^{-7}	5.0×10^{-8}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	n.d.
clindamycin	0.016	10.9	2.0×10^{-8}	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	$<5.0 \times 10^{-10}$	n.d.
S. pneumoniae strain No. 9 [49, 50]							
ciprofloxacin	16	2.4	2.0×10^{-5}	1.0×10^{-5}	$<1.7 \times 10^{-9}$	$<1.7 \times 10^{-9}$	$<1.7 \times 10^{-9}$
moxifloxacin	0.5	3.1	4.5×10^{-5}	7.0×10^{-6}	4.3×10^{-6}	2.8×10^{-7}	$<1.7 \times 10^{-9}$
erythromycin	8.0	1.7	2.0×10^{-2}	4.0×10^{-3}	2.0×10^{-4}	2.0×10^{-5}	n.d.
clarithromycin	4.0	1.5, 3.0	2.0×10^{-3}	6.0×10^{-4}	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	n.d.
clindamycin	0.03	10.9	3.0×10^{-3}	1.5×10^{-3}	1.0×10^{-7}	$<1.0 \times 10^{-9}$	n.d.
S. aureus SA079, vancomycin- and methicillin susceptible [55]							
ceftobiprole	1.0	19.0	n.d.	1.4×10^{-9}	2.8×10^{-10}	$<1.4 \times 10^{-10}$	n.d.
vancomycin	1.0	49, 63	n.d.	2.8×10^{-8}	2.8×10^{-9}	$<1.4 \times 10^{-10}$	n.d.
linezolid	2.0	8.1, 12.7, 12.9	n.d.	$<3.9 \times 10^{-10}$	$<3.9 \times 10^{-10}$	$<3.9 \times 10^{-10}$	n.d.
Q-D	0.5	3.2/7.9	n.d.	2.4×10^{-8}	3.9×10^{-9}	$<3.9 \times 10^{-9}$	n.d.
daptomycin	0.5	5.9, 6.7	n.d.	$<0.8 \times 10^{-10}$	$<0.8 \times 10^{-10}$	$<0.8 \times 10^{-10}$	n.d.
minocycline	0.125	0.76	n.d.	$<2.4 \times 10^{-10}$	$<2.4 \times 10^{-10}$	$<2.4 \times 10^{-10}$	n.d.
mupirocin	0.25	topical	n.d.	1.7×10^{-7}	3.4×10^{-9}	$<3.4 \times 10^{-9}$	n.d.

Table 2 continued..

S. aureus SA510, vancomycin- and methicillin resistant [55]							
ceftobiprole	2.0	19.0	n.d.	$<1.5 \times 10^{-10}$	$<1.5 \times 10^{-10}$	$<1.5 \times 10^{-10}$	n.d.
vancomycin	32	49, 63	n.d.	n.d.	n.d.	n.d.	n.d.
linezolid	2.0	8.1, 12.7, 12.9	n.d.	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$	$<3.3 \times 10^{-10}$	n.d.
Q/D	0.5	3.2/7.9	n.d.	3.3×10^{-6}	3.3×10^{-8}	$<3.3 \times 10^{-10}$	n.d.
daptomycin	0.25	5.9, 6.7	n.d.	2.6×10^{-7}	$<2.6 \times 10^{-10}$	$<2.6 \times 10^{-10}$	n.d.
minocycline	0.25	0.76	n.d.	$<2.5 \times 10^{-10}$	$<2.5 \times 10^{-10}$	$<2.5 \times 10^{-10}$	n.d.
mupirocin	0.125	topical	n.d.	6.1×10^{-8}	2.2×10^{-8}	6.1×10^{-9}	n.d.
P. aeruginosa PAO1 [48]**							
ceftolozane	0.5	90.2	n.d.	n.d.	$<6.1 \times 10^{-9}$	$<6.1 \times 10^{-9}$	$<6.1 \times 10^{-9}$
ceftazidime	2.0	119.1	n.d.	n.d.	4.3×10^{-7}	3.7×10^{-7}	1.2×10^{-7}
imipenem	1.0	21.6, 43.9	n.d.	n.d.	$<1.0 \times 10^{-6}$	$<6.1 \times 10^{-9}$	$<6.1 \times 10^{-9}$
ciprofloxacin	0.125	2.4	n.d.	n.d.	3.4×10^{-8}	$<6.1 \times 10^{-9}$	$<6.1 \times 10^{-9}$

and depending on its susceptibility to temporarily sub-inhibitory concentrations. However, additional aspects should be considered. Since the characterization of antibacterial effects generated by using different PK/PD models had a high level of comparability [60-66], the varying results discussed below cannot have been due to the use of different PK/PD models as such. However, emergence of resistance was time- and inoculum dependent. In contrast to endpoint measurements the time of analysis for mutant frequencies is not defined in PK/PD models. Since mutant frequencies vary in the course of PK simulations, this ought to be the case. For example, total viable counts of a MRSA test strain initially decreased and ciprofloxacin- or levofloxacin-resistant subpopulations emerged in low numbers till 12 h. However, regrowth and emergence of resistant subpopulations achieving viable counts comparable to the drug-free controls were noticed subsequently (Table 3) [67, 68]. This example demonstrates that mutant frequencies vary by several orders of magnitude not only throughout the test period of PK/PD experiments but also in comparison to the discrete endpoint method exposing the identical test strains in the same medium to constant antibiotic concentrations. Due to the workflow in the laboratory samples are withdrawn repeatedly during the first 10 to 12 hours of PK/PD experiments and then possibly at $\geq$24 hours. Thus, mutant frequencies following exposure to fluctuating drug concentrations will be quantitated at 10 to 12 hours while the discrete endpoint measurement following exposure to constant concentrations is performed at 18 to 24 hours. Furthermore, a relatively low inoculum of about 10^6 CFU/mL is used most frequently in PK/PD experiments [65] whereas high inocula of 10^8 to 10^{10} CFU/mL are used in endpoint measurements. A longer incubation period and a higher inoculum as applied in the endpoint measurements should theoretically result in higher mutant frequencies as compared to a shorter drug exposure involving lower numbers of bacteria as would be typical for PK/PD assays. However, the opposite proved to be true. This raises the following questions as to why results generated *in vitro* following exposure to constant or fluctuating drug concentrations or results generated in experimental animals differ to such an extreme extent. Recently, a workshop was held entitled "pharmacokinetics-pharmacodynamics for development of therapeutics against bacterial pathogens" [63]. The aims were to compare various PK/PD models and to identify methods for deriving and utilizing PK/PD relationships to design optimal dosage regimens for patients. It was concluded that preclinical PK/PD models are essential tools for drug development and for providing important information for dose selection in humans, whilst additionally being well suited to establish PK/PD indices and dosage regimens that best predict bacterial killing and resistance prevention *in vitro* [61-63]. However, absence of resistance emergence over the first 12 to 24 hours does not correlate with resistance prevention over 10 days, as it would be relevant for the clinical setting.

The sixth issue to be addressed is the use of different media potentially having a significant impact on the expression of antibiotic resistance. The controversial debate about the effect of zinc on carbapenem resistance is based on the observation that the steadily increasing global carbapenem resistance due to production of the metallo ß-lactamases (MBLs) was not paralleled by increasing reports about poor clinical outcome [69]. MBLs are active *in vitro* as zinc is essential for their activity because of its binding to a highly conserved motif in the active site [70-72] and as zinc concentrations are high *in vitro* ranging from e.g. 0.36 to 7.8 mg/L in 21 different brands of Mueller-Hinton Broth (MHB) [73]. However, zinc concentrations in human body fluids and tissues like urine, muscle, heart, lung, brain and placenta are low, and hence free zinc is rare within cells and body fluids [74, 75]. Also, zinc concentrations were lower in uninfected tissues of experimental animals than in serum and were undetectable in infected tissues [76]. Therefore, MBL-producing strains were resistant to carbapenems *in vitro* under routine conditions but susceptible *in vitro* in the presence of chelators. Also, carbapenem treatment of animals infected with MBL producers was effective. Although these findings were discussed controversially [77-81] they support the conclusion that routine *in vitro* susceptibility testing may not mirror pathophysiologically relevant conditions and may not correlate with clinical efficacy. This notion is supported by additional findings. Simulations of growth conditions in various infectious sites like respiratory or urinary tract

Table 3. Mutant frequencies for resistance to moxifloxacin (MXF), ciprofloxacin (CPX) and levofloxacin (LVX) in *S. aureus* (methicillin resistant strain P8) exposed either to constant fluoroquinolone concentrations of x-times their individual MICs or to fluctuating concentrations simulating serum concentrations following oral doses of 400 mg MXF once daily, 500 mg CPX twice daily and 500 mg LVX once daily. Fluoroquinolone-resistant subpopulations with 2-, 4-, and 8-times elevated MICs were quantitated during the PK-simulations. Inocula of 3.2×10^9 CFU/mL and 1.2×10^6 CFU/mL were used for exposures to either constant or fluctuating drug concentrations; positive exponents indicate growth and high numbers of resistant subpopulations (modified acc. to 67, 68).

Agent (MIC, mg/L)	**x-times MIC**	**Constant concentration**	**Fluctuating concentrations**				
			6h	**12h**	**24h**	**36h**	**≥48h**
MXF (0.062)	2	4.6×10^{-6}	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$
	4	$\leq 3.2 \times 10^{-9}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$
	8	$\leq 3.2 \times 10^{-9}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$
CPX (0.25)	2	4.6×10^{-2}	2.5×10^{-3}	1.0×10^{-2}	4.3×10^{-2}	1.5×10^{1}	2.0×10^{3}
	4	3.2×10^{-5}	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	4.3×10^{-2}	1.5×10^{1}	2.0×10^{3}
	8	2.2×10^{-4}	$\leq 1.2 \times 10^{-6}$	$\leq 1.2 \times 10^{-6}$	4.3×10^{-2}	1.5×10^{1}	2.0×10^{3}
LVX (0.12)	2	5.8×10^{-5}	1.0×10^{-5}	3.2×10^{-5}	1.6×10^{-4}	1.5×10^{1}	2.0×10^{3}
	4	7.2×10^{-6}	$\leq 1.2 \times 10^{-6}$	8.0×10^{-5}	1.0×10^{-4}	1.5×10^{1}	2.0×10^{3}
	8	4.8×10^{-8}	$\leq 1.2 \times 10^{-6}$	9.0×10^{-5}	8.3×10^{-5}	1.5×10^{1}	2.0×10^{3}

resulted in significantly increased mutant frequencies as compared to conventional assays [82-84]; furthermore, adaptive ß-lactam-, macrolide-, ketolide-, aminoglycoside-, and fluoroquinolone-resistances emerged [18, 85-91]. Expression of an efflux pump mediating antibiotic resistance, MexXYOprM, in *P. aeruginosa* was significantly higher in sputum than in laboratory media [92, 93]. In particular, real time PCR allowing analysis of bacteria directly *in situ* in patients revealed that expression of *ampC* and *mexXY* genes was 87.9- and 4.5-fold higher in cystic fibrosis patients than *in vitro*. In addition, expression of *aphA* gene coding for aminoglycoside inactivating aminoglycoside 3'-phosphotransferase was increased [94]. Highly mecillinam-resistant *E. coli* strains when grown in MHB were phenotypically reverted to antibiotic susceptibility when grown in human urine, while still maintaining the resistance mutation [95]. Derepression of ß-lactamase production during growth of *P. aeruginosa* in plasma or ascetic fluid was lower than in MHB but higher in urine [96]. While development of tobramycin resistance in conventional laboratory medium, in synthetic CF-sputum, and in human urine was similar, resistance to ceftazidime and ceftazidime-avibactam differed significantly in these three media being highest in conventional medium, moderate in synthetic CF-sputum, and low in urine [97].

Furthermore, different commercial media yielded distinct results. Mutant frequencies to kanamycin resistance differed more than 100-fold on Luria- and Nutrient agar [98]. Expression of amoxicillin-clavulanate resistance and fitness of various antibiotic-resistant *E. coli* strains differed in three commercially available media and in particular in human urine and urothelial organoids [99]. Antibiotic resistance emerged in the absence of any antibiotic and without any known common denominator between stressor(s) and antibacterial agents in four different media to variable degrees during serial passages. Genetic adaptations with mutations in the respective resistance genes led to ß-lactam, aminoglycoside, quinolone, tetracycline, glycopeptide, polymyxin, chloramphenicol and rifampicin resistance in Gram-positive and Gram-negative species [100]. In addition, patterns of mutational vancomycin tolerance in MRSA differed in strains either grown in CA-MHB or Roswell Park Memorial Institute medium (RPMI), a mammalian cell culture medium that mimics human physiology better. Sequencing of vancomycin-tolerant clones revealed that mutations overlapped on two cell wall regulons but were media-specific in genes affecting cell surface charge and proton motive force. The emergence of vancomycin tolerance in RPMI medium was not paralleled by emergence of vancomycin tolerance in CA-MHB and thus passes undetected under standard routine conditions [101]. Hence, the choice of commercially available media, artificial or genuine body fluids, and humanized media or organoids has far reaching consequences since the categorization of an isolate into either the susceptible or resistant state as well as mutant frequencies and expression of resistance genes is variable and may even be contradictory. However, neither the comparability of routine *in vitro* data with those using humanized media or body fluids, or *ex vivo/in vivo* models has yet been evaluated in direct comparisons, nor has the predictability of preclinical data for emergence of resistance and thus treatment failures in patients ever been assessed. Thus, “one size does not fit all”, i.e. antimicrobial susceptibility testing and quantification of mutant frequencies by using predominantly MHB or CA-MHB does not reflect antibiotic activity or emergence of resistance in different pathophysiologically relevant environments. Furthermore, data quoted above do not allow generalizing conclusions such that mutational or adaptive resistance is either more or less likely under *ex-/in vivo* than under *in vitro* conditions.

Although endpoints characterizing resistance development are well defined by regulatory authorities, methods and materials used by academia and pharmaceutical industry vary widely. The lack of standardization offers intentionally or unintentionally many degrees of freedom for creative design of experiments generating variable results. Consequently, methods and materials used should be standardized and should not only take statistical principles into account but should also consider the pathophysiology of the respective infectious site. Possibly one could imagine following a twin track approach, i.e. mutant frequencies could be quantitated in the usual routine procedure to generate some basic parameters and to maintain

comparability with previous studies; the use of a specific medium and of quality control strains in analogy to MIC testing would be desirable. In a parallel or consecutive approach modified media simulating the pathophysiologically relevant conditions at the infectious sites or *ex-/in vivo* methods could be used – ideally in combination with PK/PD studies. Furthermore, the cause(s) for the differences between static and dynamic *in vitro* models as well as *in vivo* models should be clarified. Hypothetically, the various aspects reviewed below may have an impact on resistance development *in vivo* thus possibly being relevant for the design of novel methods and models.

4. Bacterial metabolism and antibiotic resistance

Regulatory authorities request information about the mode(s) of action, mechanism(s) of resistance, and the potential for cross-resistance [42]. It is presumed that modes of action and/or mechanisms of resistance do not differ under *in vitro, ex/in vivo*, and clinical conditions. A target or a resistance mechanism identified under *in vitro* conditions is considered to be expressed in an identical structure *in vivo* as well. This assumption has so far not been scrutinized. However, physiological studies [102-105], proteome [106-112] and metabolome profiling [113-119], as well as transcriptomics [120, 121] have revealed that the physiology of bacteria grown *in vitro* and *in vivo* differs significantly.

4.1. Central carbon metabolism and antibiotic resistance

A plethora of preclinical data has indicated that environmental conditions have a significant impact on the categorization of pathogens into their susceptible or resistant state [33, 116-132]. Conversely, the acquisition of antibiotic resistance may result in changes of bacterial metabolism [116, 117, 122, 132-138]. Bacteria growing under physiologically relevant conditions acquire energy from varying carbon sources. In general, a key regulatory mechanism controlling metabolic adaptations is carbon catabolite repression (CCR). CCR regulates gene promotor activity in *Enterobacteriaceae* either by inducer exclusion or by cyclic AMP (cAMP) binding to the catabolite activator protein (CAP). The cAMP-CAP complex binds to DNA, thus activating the promoters of about 200 genes [139-142]. In Pseudomonads, however, CCR operates at the mRNA level. RNA chaperone protein Hfq binds directly to the 5' end of the mRNA thus repressing gene expression [143-145]. In *B. subtilis* and other Gram positive bacteria with low GC content CCR is attained by the global transcription regulator CcpA, which is activated by the availability of its phosphorylated cofactors. Phosphorylation of these proteins is catalysed by the metabolite-controlled kinase HPrK/P [146, 147].

Adaptive resistance of *E. coli* to penicillin G, ampicillin, mecillinam [148-152], and fosfomycin [152-156] and vice versa increased fosfomycin activity under anaerobic conditions [157, 158] and adaptive resistance to colistin and polymyxin [152] nalidixic acid [150], streptomycin [131, 152, 159, 160], trimethoprim and several sulfonamides [131, 152], as well as tetracycline [152] was affected by cAMP. Resistances were most frequently due to impaired uptake [152, 161]. Furthermore, genes expressing penicillin acylase [149] and chloramphenicol acetyl transferase in *E. coli* [162-166] are under cAMP control. The pore-forming antibiotic colicin targets the outer membrane protein CirA of *P. aeruginosa*. The RNA chaperone Hfq acts as the repressor of *cirA* mRNA translation thus preventing translation initiation [167]. Furthermore, *hfq* deletion strains show increased susceptibilities to cefepime, imipenem, gentamicin, ciprofloxacin, fosfomicin, colistin, and tetracycline [168]. Transcriptome analysis revealed that Hfq affected antibiotic transport, carbon metabolism, and cell wall composition. These data are well in agreement with the finding that the global regulator Crc [133] as well as various carbon sources [169] modulate antibiotic susceptibilities in *P. aeruginosa* [133]. Penicillin tolerance in streptococci was almost totally restored *in vitro* as well as in an experimental endocarditis model by *ccp*A deletion [170]. Inactivation of *ccpA* altered sensitivity of *S. aureus* to fosfomycin and ampicillin in the absence of exogenous glucose-6-phosphate [171]. Deletion of *ccpA* increased oxacillin susceptibility in a highly methicillin-resistant *S. aureus* strain and reduced teicoplanin resistance in a glycopeptide-intermediate-resistant *S. aureus* strain [172, 173]. Single species cultures of *S. aureus* and *P. aeruginosa* as well as their co-culture in the L929 cell line model revealed that the *ccrp* expression

level increased two-fold paralleled by an amikacin resistance development in the co-culture model as compared to the mono-cultures, whereas amikacin susceptibilities increased three- to four-fold as *ccrp* expression levels declined [174]. CcpA was found to be essential for the replication of *S. aureus* in a murine model of staphylococcal abscess formation [175]. The *in vitro* wound model study as well as data generated in the experimental endocarditis- or abscess models demonstrate that CCR is relevant *in vivo*, too. These data demonstrate that in the absence of environmental stressors the carbon source used has a significant impact on the emergence of adaptive antibiotic resistance and expression of resistance genes, too.

Furthermore, the bacterial physiology as such, i.e. the metabolic state of the bacterium, affects resistance development. Mutations in genes coding for enzymes of the central carbon metabolism caused fosfomycin resistance in a *S. maltophilia* strain not harbouring any of the known fosfomycin resistance mechanisms and growing in the absence of any antibiotic [176]. Inhibition of cellular respiration in *P. aeruginosa* induced phenotypic tobramycin tolerance although tobramycin import was not reduced [176]. Conversely, activation of central carbon metabolism augmented tobramycin activity [169]. Furthermore, GC-MS-based metabolomics revealed that central carbon metabolism was repressed in cefoperazone/ sulbactam-resistant *P. aeruginosa* [177]. These data indicate that targeted and well-planned interventions on metabolism may open up the possibility to convert resistant bacteria into susceptible ones [132, 178-180]. Thus, metabolism and antibiotic resistance are associated [118], such that not only classical resistance mutations but also mutations in metabolic genes could be relevant for resistance development [117, 176]. However, the impact of bacterial metabolism and CCR on the emergence of resistance and antibiotic susceptibilities of pathogens has so far not been considered systematically due to a focus on traditional parameters but ought to be taken into account during drug development in future. A significant and clinically relevant aspect of antibiotic resistance development will otherwise be disregarded.

4.2. Growth phase and growth rate as neglected drivers of antibiotic resistance

Bacterial central carbon metabolism is closely linked to parameters like nutrient availability, growth rate and growth phase [181-184]. Growth *in vitro* in nutrient rich media, *ex vivo* in tissue homogenates or *in vivo* in experimental animals affects generation times. For example, generation times of the same *E. coli* strain growing either in MHB, blood or kidney homogenate, or in an animal sepsis- or pyelonephritis model increased from 0.4 h (MHB), 0.6 h and 0.8 h (blood and kidney) to 1.0 h in the sepsis model and 2.9 h to 3.5 h in the pyelonephritis model [103-105]. The generation times of pneumococci causing experimental meningitis in afebrile rabbits were 1.1 h, whereas doubling times in febrile animals were 2.76 h as compared to 0.61 h *in vitro* [185]. Analogues data were generated for *H. influenzae* in an identical model [186]. Generation times of *P. aeruginosa* in broth and in a thigh infection model were 0.4 h and 0.9-2.2 h, respectively, and generation times of *S. aureus* growing *in vitro*, colonizing the human nose or causing osteomyelitis were 0.4 h, 3.4 h and 8-24 h, respectively [103-105, 187, 188] and doubling times of dental microbes were 4.8 h [189]. Analogous data were obtained in a mouse peritonitis model [190] and in patients suffering from urinary tract infections [191] in which/whom bacterial growth rates were probed by *E. coli* chromosome replication using quantitative PCR [192]. Likewise, estimation of evolutionary changes in bacteria revealed that generation times in natural habitats ranged from 1.1 h for *V. cholerae* to 25 h in *S. enterica* as compared to 0.6 h and 0.5 h *in vitro* [193, 194]. Thus, bacteria cultivated *in vitro* under routine growth conditions in nutrient-rich media at neutral pH grow rapidly and live in cosy environments whereas pathogens causing infections in complex *ex vivo* or *in vivo* models in mostly acidic and nutrient-poor habitats live in hostile environments. Growth rates of bacteria growing under pathophysiologically relevant conditions correspond to those of the transition phase from the late logarithmic to the stationary growth phase under routine *in vitro* conditions. This gives reason to assume that emergence of resistance may be affected by slow growth.

Gene expression was found to be growth phase dependent [195-198]. Expression of chloramphenicol acetyltransferase (cat) in *E. coli* increased about 11-fold as growth rates decreased 5- to 6-fold due to stabilization and thus accumulation of cat mRNA [199-201].

In general, growth phase transitions and slow growth were associated in particular with changes in mutant frequencies increasing by $\geq$ one order of magnitude [19, 22, 202-223]. The Mutation frequency decline protein Mfd is a highly conserved transcription-coupling repair factor involved in the repair of DNA lesions caused by e.g. UV light or exogenous reactive oxygen species (ROS) and nutritional stress or growth *in vivo*. Recent evidence has demonstrated that Mfd affects the global bacterial transcription profile. Mfd increased not only mutagenesis but also enabled bacteria to evolve antibiotic resistance in the absence of any antibacterial agent [224-227]. Mfd promoted emergence of fluoroquinolone-, rifampin-, fosfomycin-, trimethoprim-, kanamycin- and vancomycin-resistance in broth-grown Gram-negative species including *C. jejuni* lacking the SOS response, Gram-positive bacteria, and non-fermentors as well as *S. typhimurium* or *S. enterica* during infection of eukaryotic cells in the absence of any antibiotic. Mfd also promoted the evolution of hypermutators. Vice versa, strains lacking Mfd had a 2- to 5-fold decrease in mutation rates compared to wild-type strains [224-227]. However, inactivation of Mfd in *S. aureus* resulted in biofilm formation [228] whereas Mfd increased antibiotic susceptibility of *H. pylori* [229]. In addition, wild type E. coli or its isogenic hypermutator genotype colonizing the mouse gut were characterized by up to three orders of magnitude higher mutant frequencies and mutation rates as well as mutation rate polymorphism as compared to the ancestral genotypes grown *in vitro* [230-232]. Analogous data were generated for adaptation of *S. typhimurium* to mice [233]. These data demonstrate that slow growth *in vitro* as well as in experimental animals affected gene expression resulting in promotion of mutagenesis coupled with the evolution of antibiotic resistance in the absence of any antibacterial agent. It therefore may not come as a surprise that mutant frequencies for fluoroquinolone resistance *in vivo* during treatment of experimental infections were high. Treatment of pyelonephritis with 8 different fluoroquinolones resulted in an emergence of resistant post-treatment isolates of *P. aeruginosa* at a mean rate of 3.5×10^{-1} [234-240]. Ciprofloxacin- and pefloxacin resistant mutants emerged at rates of 6.1×10^{-1} and 7.7×10^{-1}, respectively in a peritonitis model [234, 237] and at a rate of 3×10^{-1} in an endocarditis model [238-240] as compared to i*n vitro* rates ranging from 10^{-7} to 10^{-9} for *E. coli* and 10^{-5} to 10^{-7} for *P. aeruginosa*.

Several reviews have summarized the impact of environmental stressors on mutant frequencies in general [241, 242] and bacterial stress responses as determinants of resistance to antibiotics in particular [243-250], and the reader is kindly referred to these publications. Only those stress and growth phase-induced adaptations leading to target or efflux pump modifications in the absence of any antibacterial agent are summarized in Table 4 [251-296]. DNA supercoiling and the production of ribosomes and thus transcription and translation largely cease when bacteria transit from the late logarithmic to the stationary phase [296-306]. One of the essential modulators of bacterial physiology in response to environmental stress is guanosine 5'-triphosphate-3'-diphosphate [(p)ppGpp] or guanosine-3'-5'-bis diphosphate (ppGpp) [307, 308]. In *E. coli*, growth rate is inversely proportional to intracellular ppGpp concentrations, thus demonstrating that ppGpp is involved in growth rate control and gene expression during slow growth of bacteria. In addition, the so-called superhelicity monitors like Factor for Inversion Stimulation protein (FIS) and cAMP receptor protein decrease during slow growth [302, 309], thus indicating that (p)ppGpp or ppGpp, *fis* and *crp* jointly regulate negative superhelicity and protein synthesis. FIS directly accelerates growth phase-specific superhelicity of DNA in *E. coli* [310]. In addition, FIS affects DNA superhelicity indirectly by repressing transcription of *gyrA* and *gyrB* [311] which were also repressed approximately 5-fold by (p)ppGpp during stationary phase [312]. Overall, the accumulation of (p)ppGpp causes the differential expression of approximately 500 genes [313], and hence it is not surprising that (p)ppGpp or ppGpp

Table 4. Stress-induced and antibiotic-independent modifications of bacterial cell structures and/or metabolism leading to target or efflux pump modifications (PBP=penicillin binding protein; (p)ppGpp=guanosine 5'-triphosphate-3'-diphosphate or ppGpp=guanosine-3'-5'-bis diphosphate; MEC= mecillinam; MEM=meropenem; CLX=cloxacillin; LPS=lipopolysaccharide; SOD=superoxide dismutase; ROS= reactive oxygen species; n.d.=not done).

Stressor	Gene/protein affected	Effect	Reference
E. coli			
C-, P-, and N-starvation	*stiC* = starvation-inducible, σs -dependent 2 gene locus *yohC* + *pbpG*	Augmented synthesis of YohC = putative transport protein + PbpG = PBP 7/8. *yohC* + *pbpG* are co-expressed during first few hours of stravation, but later become expendable	[251-253]
Aminoacyl-t-RNA synthase mutations	p^{tac}-*relA* conferring MEC resistance	PBP2 is dispensable in MEC resistant mutants overexpressing ppGpp which may regulate the transcription of a gene being involved in MEC resistance	[254-256]
Fe limitation	SpoT synthesizing and hydrolysing (p)ppGpp	Fe limitation increased ppGpp concentrations leading to an expression of Fe uptake genes, thereby alleviating Fe limitation but conferring MEC resistance. This MECr phenotype is growth rate independent.	[257]
Amino acid starvation	*spoT*	(p)ppGpp acts as a coordinator of cell growth in response to reduced LPS and ADP synthesis. Signals for perturbations in these pathways are relayed via SpoT. LPS depletion resulted in reduced growth rates and resistances to cell wall active antibiotics and fluoroquinolones.	[258]
pH	*rpoS* (coding for σs)	PBP1a activity is reduced in acidic conditions, rendering the cell reliant on PBP1b for cell wall integrity and viability. Cells displayed an increase in intrinsic resistance to PBP2 and PBP3 targeting antibiotics, i.e. mecillinam and cephalexin, whereas susceptibility to ampicillin and cefsulodin was not pH dependent.	[259, 260]
Microcin J25	*yojI*	Resistance to microcin J25 was directly correlated to ppGpp accumulation via induction of YojI, a chromosomally encoded efflux pump expelling microcin J25 from cells	[261]
Intraleukocytic growth	n.d.	Loss of penicillin binding activities of PBPs 1a,b, -2, -3, -4, -5. -6, and -7a,b. Human myeloperoxidase inactivated in particular PBPs3, -4, and -7b	[262, 263]
Temperature	*rpoS, rpoH* (coding for a heat shock regulator protein)	Temperature >37°C reduced stability of PBP3 (other PBPs have not been studied)	[264]
Slow growth, anaerobiosis	*mdtEF* (multidrug efflux pump)	*mdtEF* promoter activity greatly increased with cell growth and reached the maximum level at the late stationary phase conferring drug tolerance to *E. coli* at the stationary phase as well as anaerobiosis whereas the expression of *acrAB, emrAB, emrD, emrE, emrKY, mdfA,* and *ydgFE* is stable at moderate levels during any growth phase.	[265]

Table 4 continued..

Osmotic stress	Heat-stable nucleoid structuring (H-NS) protein affecting levels of RpoS	H-NS coordinates the expression of over 200 genes with a large number involved in both bacterial virulence and drug resistance.	[266]
P. aeruginosa			
Mouse lung infection	*dacB* in the mouse model strain. *oprD, mexB, mexY, ampD, dacB* in the clinical isolates	Inactivation of the *dacB*-encoded nonessential PBP4, which behaves as a trap target for ß-lactams. In addition, AmpC and the specific activation of the CreBC (BlrAB) two-component regulator, which in turn plays a major role in resistance, were overproduced. Similar findings were generated in pan ß-lactam resistant clinical isolates, which were characterized by significantly reduced binding affinities of imipenem to PBP2 and of ceftazidime, ceftolozane, imipenem to PBP3 in addition to AmpC, efflux pumps, and OprD,	[267, 268]
Intraleukocytic growth	n.d.	Human myeloperoxidase inactivated in particular PBPs1a,b, -2, -3, -4, and -5,	[263]
SOD	*sodB, relA, spoT*	SOD activity directly or indirectly contributed to the alterations in the cell envelope thus becoming less permeable during stationary phase, so that drug penetration was limited,	[269, 270]
ROS	*relA, spoT*	High concentrations of (p)ppGpp were associated with significantly reduced activities of ofloxacin, meropenem, colistin, and gentamicin, which was reverted in (p)ppGpp^0 mutants	[269]
ROS	*mexXY*	Induction of MexXY-OprM multidrug efflux pump via induced expression of PA5471 (a protein whose expression is promoted by ribosome-disrupting antimicrobials) thus mediating aminoglycoside resistance in vivo. ROS did not enhance aminoglycoside resistance in vitro. Other dug classes were not tested.	[271]
A. baumanii			
Mutants overexpressing (p)ppGpp	*abeB, tet(A), adeB, adeI, adeJ and adeK*	Reduced expression of efflux pump related genes *abeB, tet(A), adeB, adeI , adeJ and adeK* resulting in decreased activities of gentamicin, tetracycline, erythromycin and trimethoprim	[272]
V. cholerae			
ROS	*acnB, fbpA*	(p)ppGpp suppressed tricarboxylic acid cycle by repressing the aconitase B encoding gene *acnB* and repressed expression of iron (III) ABC transporter substrate-binding protein FbpA due to (p)ppGpp-accumulation, resulting in a reduction of intracellular free iron, required for ROS-generation thus reducing oxidative stress and preventing cell death.	[273]
H. influenzae			
Rat tissue chambers	n.d.	Growth in animals caused a rapid change to a physiological state similar to that found in late-stationary-phase cultures in vitro. Affinities of PBPs 2, -3A,B, -4, and -6 for penicillin were reduced paralleled by a reduced peptidoglycan synthesis. In vitro growth in peritoneal- or cerebrospinal fluid yielded identical results.	274, 275

Table 4 continued..

Temperature	*ponB, acrR, ompP2, acrB*	Synthesis of PBP1b and AcrAB-TolC efflux pump were significantly increased at 42°C as compared to 37°C. In contrast, synthesis of outer membrane protein P2 and AcrB were significantly lower.	[245, 246]
S. enterica			
C starvation	*stiC* = starvation-inducible, σs -dependent 2 gene locus *yohC* + *pbpG*	Augmented synthesis of YohC = putative transport protein + PbpG = PBP 7/8. *yohC* + *pbpG* are co-expressed during first few hours of stravation, but later become expendable	[276-280]
C starvation	*aadA*	Increased expression of aminoglycoside-adenyl-transferase thus increasing aminoglycoside resistance. Regulated by (p)ppGpp	[281]
Intracellular growth	PBP2 and -3 paralogue genes coding for $PBP2_{SAL}$ and $PBP3_{SAL}$	Production of alternative $PBP2_{SAL}$ and $PBP3_{SAL}$ replacing PBPs 2 and -3 and binding current ß-lactams with low affinity. The alternative PBPs are expressed and promote cell division only in the acidic intraphagosomal environment as well as in liver and spleen.	[282-284]
S. aureus			
Enzymatic attack	n.d.	Human myeloperoxidase inactivated in particular PBPs 1, -2, -3, and -4	[263]
Intraleukocytic growth	n.d.	Total numbers of PBP2 were significantly reduced (285, 286) or even totally eliminated (287) and activity was completely inactivated, whereas total numbers of PBP4 increased	[285-287]
Rat tissue cages	n.d.	Antibacterial activities of cefazolin and cefotaxime binding selectively to PBPs2 and -4, and PBPs1 and -2, respectively, were significantly reduced as compared to cephalexin binding to PBPs 1 and -3	[285, 286]
Bicarbonate	*mecA, sarA*	Bicarbonate ($NaHCO_3$) increases β-lactam susceptibility of $NaHCO_3$-responsive MRSA strains due to reduced synthesis of PBP2a and the global virulence gene regulator.	[288, 289]
Acidic pH and intracellular growth	*mecA* and PBP2a	MRSA phenotype reverts to MSSA. PBP 2a most likely evolved for its physiological function at pH 7 or higher by adopting a closed conformation, which is not maintained at acidic pH.	[290, 291]
Acidic pH and intracellular growth	*mecA* and PBP2a	intraphagocytic MRSA strains growing in THP-1 macrophages were as sensitive to MEM and CLX MSSA. Restoration of susceptibility of intraphagocytic of MRSA to MEM and CLX was due to the acidic pH prevailing in phagolysosomes and was mediated by an enhanced binding to penicillin-binding proteins.	[292]
Multiple species			
Slow growth	*gyrA, gyrB*	Copy numbers of gyrA and/or gyrB mRNA of *E. coli* during the log-phase were 300-times higher than during the stationary phase. In analogy, *gyrB* copy numbers of *L. pneumophila* were 10- to 100-times higher during the log-phase.	[293-295]
Slow growth	*gyrA, gyrB*	The level of supercoiling decreased as cells entered into stationary phase.	[296]

was the essential driver of target modifications summarized in Table 4. Recent reviews have summarized the direct and indirect associations between increased (p)ppGpp concentrations and antibiotic resistance, antibiotic tolerance, persistence and biofilm formation [314-318]. Among other proteins ppGpp and cAMP bind to the DNA binding transcriptional regulator BolA with strong affinity. The *bolA* gene is under the control of the alternative sigma factor "stationary phase δs", encoded by the *rpoS* gene and is induced during transition from the logarithmic to the stationary growth phase. Stressors like extremes of temperature, acidity, carbon starvation, high osmolarity and ROS as well as reduced growth rate and high cell density trigger expression of bolA. The *bolA* gene regulates amongst others factors *dacA* and *dacC* genes coding for D,D-carboxypeptidases PBPs 5 and -6. BolA overexpression reduced sensitivity to detergents and vancomycin, affected outer membrane protein accessibility and β-lactamase AmpC and was involved in the modulation of the OmpF/OmpC balance [319-324].

The most noticeable adaptations to slow- or intracellular growth *in vitro* and growth in experimental animals were reduced or suppressed synthesis of drug targets. For example, ß-lactams are characterized by their selectivity and affinity to wild type as well as mutated PBPs as ß-lactam resistance is – apart from enzymatic inactivation or import and export mechanisms - strongly associated with sequence variations of PBPs [325-334]. These data were generated in exponentially growing bacteria. However, PBP expression *in vivo* in slowly growing bacteria is frequently not detectable [251-255, 259-261, 263, 267, 268, 274-280, 282-304, 307, 308]. Mecillinam and zidebactam, for example, bind preferentially to PBP 2, which is anyway expressed in low numbers in bacteria grown under routine conditions but almost absent in stressed bacteria. Thus, mecillinam's and zidebactam's target is only minimally expressed, if at all, under pathophysiologically relevant growth conditions. But nevertheless, both agents were highly effective in various infection models and clinically. Ceftriaxone binds preferentially to PBPs 2 and 3 [327-330], which are not expressed by *S. enterica* growing intracellularly. Instead, $PBP2_{SAL}$ and $PBP3_{SAL}$ are produced in response to an acidic pH and/or nutrient limitation, this being typical for an intraphagosomal environment and growth in liver and spleen. However, ceftriaxone did not bind to these alternative PBPs. Consequently, ceftriaxone exhibited negligible *in vivo* efficacies in a mouse model or intraleukocytic growth but high *in vitro* activities under routine conditions [282-284]. This dichotomous target affinity and thus antibacterial efficacy of ceftriaxone will pass undetected if merely routine methods are applied. Further analogous findings are summarized in Table 4. The dichotomous efficacy of ß-lactams, i.e. *in vitro* activity or *in vivo* efficacy despite the target being missed, suggests that ß-lactams do not only inhibit PBPs but are likely to interact with additional targets alternative to or downstream from PBP inhibition [324, 325]. Furthermore, methicillin-resistance vanished when methicillin-resistant staphylococci were incubated in an acidic and intracellular environment instead of a neutral or alkaline pH [290, 291]. Reversion of MRSA phenotype to MSSA was due to conformational changes of PBP 2a mediating methicillin resistance at pH 7 or higher by adopting a closed conformation, which is not maintained at acidic pH, such that e.g. meropenem and cloxacillin susceptibility of intraphagocytic MRSA was restored due to enhanced binding of ß-lactams to modified PBPs at an acidic pH [293].

Expression of DNA gyrase is growth phase dependent [303]. Gene expression of *gyrA* and/or *gyrB* in *E. coli* [283, 284] and *L. pneumophila* [295], respectively, was 300-times higher during the logarithmic than during the transition from the logarithmic to the stationary phase and not detectable during the stationary phase. These findings comply well with the superhelicity of DNA [311] affected by FIS, and (p)ppGpp repressed *gyrA* and *gyrB* gene transcription [312] during stationary growth phase. The integrity of the gyrase protein synthesized during the exponentional growth phase was maintained during the stationary phase as enzyme activity is essential to reinitiate growth despite undetectable gyrase gene expression during this growth phase [296]. Consequently, ciprofloxacin targeting preferentially DNA gyrase of *Enterobacteriaceae* caused progressively slower rates of DNA damage during the stationary phase than during their exponential growth phase [296, 335]. But

nevertheless, ciprofloxacin and other fluoroquinolones interacting preferentially with DNA gyrase were and still are clinically effective which may likely be due to additional targets with which fluoroquinolones interact independent of inhibition of bacterial type II topoisomerases and irrespective of whether bacteria grow rapidly or slowly [336]. Analysis of an antisense RNA that is encoded in *cis* to the *parC* gene coding for the A-subunit of topoisomerase IV revealed that it was expressed mostly in the stationary growth phase and protected the topoisomerase IV mRNA from inactivation by endonucleases [337]. This finding indicates that synthesis of topoisomerase IV during the stationary phase was not prevented.

Growth phase changes have a minor effect on the overall fatty acid biosynthesis whereas fatty acid composition varies significantly during the growth phases. Nevertheless, this topic should be discussed here, as regulation of bacterial enoyl-acyl-carrier protein reductase (FabI), being the major enzyme of the bacterial fatty acid synthesis (FAS II) system, represents another example for conditional essentiality. Inhibition of bacterial fatty acid synthesis seemed to be attractive as it is highly conserved and considered to be essential. In addition, long-term use of fatty acid biosynthesis inhibitors isoniazid as well as triclosan seems to have validated this target. Triclosan and afabicin (AFN 1720; AFN-1252-prodrug) target specifically FabI which is the sole form of enoyl-acyl carrier protein reductase in *Staphylococcus spp*. and alternative or rescue pathways have so far not been identified [338-340]. FabI has also been detected in *E. faecalis, B. subtilis, B. anthracis, F. tularensis, Salmonella spp., M. catarralis, H. influenzae, A. baumanii, P. aeruginosa, B. pseudomallei, P. falciparum, and T. gondii*. Afabicin is considered to be a selective inhibitor of *S. aureus* although FabI is widespread among bacterial species. The FAS II system is species specific as FabI may be replaced by other carrier proteins like FabK in *S. pneumoniae* and *Lactobacillus spp*. or FabV in *V. cholerae*, or both FabI and FabK may be present in e.g. *E. faecalis* and *P. aeruginosa* [341] but triclosan is still active against those pathogens in which FabI is missing. The essential nature of the FAS II pathway has been discussed controversially as fatty acid synthesis inhibitors could in principle be antagonized by exogenous fatty acids that are abundant *in vivo*. However, FAS II inhibition in *S. aureus* could be antagonized by exogenous fatty acids as shown in some studies whereas others got opposite results [342-346]. Furthermore, inhibition of ß-ketoacyl-ACP synthase II (FabF), an essential enzyme in both *S. aureus* and *S. pneumonia,* by platensimycin (a natural FabF inhibitor) could be antagonized by exogenous fatty acids resulting in increased MICs and growth inhibition of *S. aureus* whereas MICs and growth of *S. pneumoniae* remained unaffected [339]. These differential effects are due to feedback mechanisms triggered by exogenous fatty acids. Intracellular malonyl-CoA concentrations are essential for the FAS II system. Generation of malonyl-CoA through carboxylation of acetyl-CoA by acetyl-CoA carboxylase (ACC) is affected by exogenous fatty acids. While exogenous fatty acids did not decrease intracellular concentrations of malonyl-CoA in *S. aureus*, they significantly decreased malonyl-CoA concentrations in *S. pneumoniae* [339]. Consequently, bacteria controlling fatty acid synthesis by feedback inhibition of ACC and malonyl-CoA synthesis triggered by exogenous fatty acids are able to bypass FAS II inhibition. In contrast, FAS II inhibitors remain active in the presence of exogenous fatty acids if they do not affect this feedback system. However, MICs of afabicin for *S. aureus* increased four-fold in the presence of exogenous fatty acids [339] and membrane plasticity of *S. aureus* growing in host environments led to afabicin treatment failures in experimental animals [344, 347] despite the lack of this feedback system in *S. aureus*. Likewise, the addition of fatty acids to the medium used for simple susceptibility testing revealed that triclosan resistance in clinical isolates of *S. aureus* increased by 58%. Moreover, non-cultivable triclosan-resistant fatty acid auxotrophs, which passed unnoticed under routine conditions, were detected in primary patient samples [348]. Furthermore, serum overcame FAS II inhibition by triclosan in lactobacilli and *S. agalactiae*. These findings demonstrate that the fatty-acid-dependent strategy of resistance to FAS II inhibitors is frequent and that this adaptive resistance mechanism is clinically relevant. These adaptive resistance mechanisms remain undiscovered as long as routine methods are applied. Although

these studies currently leave many questions unanswered, data indicate that several bacterial species have developed strategies to bypass FabI inhibition. These findings also indicate that not only biocides like triclosan interacting with multiple targets but also biocides like afabicin specifically inhibiting FabI may interact with additional targets [349] thus enabling clinical efficacy.

The clinical relevance of conditional or temporal essentiality of drug targets or resistance determinants has been described for a few drug-bug associations. Tolerance or even resistance of mycobacteria to isoniazid (INH) is growth phase dependent. The prodrug INH is activated by the multifunctional catalase-peroxidase KatG, which also protects against reactive oxygen and nitrogen intermediates produced by phagocytes. *katG* is expressed *in vitro* only late and in infected macrophages only upon extended intracellular growth. The mycobacterial DNA-binding protein 1 (MDP1) controlling a variety of cellular functions and long-term survival of *Mycobacterium spp.* is increasingly expressed during the stationary phase and down regulates *katG* expression thus conferring growth phase-dependent INH tolerance [350-355]. Furthermore, expression of dihydrofolate reductase, tetracycline efflux genes *tetA* and *tetR* and daptomycin resistance due to a point mutation in the putative inorganic phosphate transporter gene *pitA* were found to be growth phase dependent [356-358]. Consequently, tetracycline- and daptomycin resistances vary with time. Thus, conditional essentiality of drug targets and resistance mechanisms are clinically relevant phenomena.

These examples demonstrate that routine methods used to characterize drug targets and modes of action or mechanisms of resistance do not mirror the impact of physiologically relevant growth conditions on the essentiality of drug targets or the emergence of mutational or adaptive resistance. Therefore, methods used to characterize modes of action and mechanisms of resistance as well as quantification of mutant frequencies should reflect pathophysiologically relevant conditions. Such modifications should mirror substantial adaptations of bacteria to growth in hostile as opposed to cosy environments. They should integrate stress stimuli affecting expression of additional or alternative drug targets and mutational or adaptive resistance such as in particular an acidic environment [259, 260, 282-284, 290, 291], increased temperature [267, 276, 277], and slow [265, 293-296], or intracellular growth [262, 263, 282-287, 290, 291] etc. prevailing in infected patients.

Furthermore, the temporal and conditional essentiality of targets and resistance mechanisms leads to the question of whether the currently common target-based screening methods are effective. Conversely, the currently common use of strains with various target mutations in order to analyse antibacterial spectra and cross-resistances may be incomplete. Consequently, the question should be addressed if the current assumption of a monocausal relationship between drug target or resistance determinant and drug activity is still adequate and sufficient. A further issue is whether a holistic view should be adopted, integrating natural diversity and variability as well as complex regulatory and communication networks into the design of experiments analysing the modes of action and mechanisms of resistance under clinically relevant conditions.

5. Induction of antibiotic resistance by non-antibiotics

Another driver of adaptive acquisition of antibiotic resistance is the so-called non- or un-specific induction of resistance, which, however, represents a specific trigger of resistance development due to subtle structural similarities between the antibiotic and the stressor. Based on observations that high ß-lactamase concentrations were detected in infected but untreated animals in kidney homogenates of *S. aureus*-infected mice, and in the granuloma pouch model infected with *P. mirabilis* [359, 360] it was proven that body fluids like serum, exudate and cerebrospinal fluid as well as the amino acids L-cysteine and L-valine, aromatic amino acids and also haemine, thiamine and folic acid induced expression of ß-lactamases in *E. coli, P. aeruginosa, E. cloacae, S. marcescens* and *C. freundii* in the absence of any antibiotic [361-366]. The inductive potential of L-cysteine, L-valine and L-phenylalanine may likely to be due to their role as precursors of penicillin thus having some structural relationship

to the ß-lactam nucleus. L-valine and L-cysteine plus L-α-aminoadipic acid are condensed to δ-L-α-aminoadipyl-L-cysteinyl-D-valine and phenylalanine is incorporated into the side chain precursor phenylacetate [367-370]. Inducers and ß-lactams have the bicyclic structure in common, whereas monocyclic compounds lacked induction potency. Loss of the aliphatic residue of tryptophan was linked with the loss of ß-lactamase induction potency. Unspecific induction of AmpC ß-lactamase in *E. coli* was dependent on intact *ampG* function. Mutated and thus unfunctional *ampG* alleles were characterized by a change from glycine to aspartate [366] Furthermore, glycine [371-374] and many D-amino acids [375, 376] induced ß-lactamase production in an antibiotic-free medium. Glycin is one of the peptidoglycan precursors and D-amino acids were incorporated into the peptidoglycan through a direct extra-cytoplasmic transpeptidation. These reactions take place in particular in slowly growing and resting bacteria through two extra-cytoplasmic pathways but not through a cytoplasmic incorporation into a D-Ala-D-Ala dipeptide precursor. Incorporation of D-amino acids into peptidoglycan altered crosslinking and the total amount of peptidoglycan per cell but growth and morphology were not affected [371, 377-380]. Altered synthesis and/or damage of peptidoglycan due to incorporation of D-amino acids may theoretically trigger a regulatory cascade possibly linked to the known peptidoglycan recycling pathway and an induction of AmpC ß-lactamase. This hypothesis has not yet been addressed systematically. D-amino acids and enzymes involved in their metabolism are ubiquitous in nature and are used in agriculture, food industry and biomedicine [381], so that intake with food and thus deployment of their ß-lactamase inductive potential are physiologically relevant and may represent a real threat.

Likewise, resistance to fluoroquinolones was induced by antibacterially inactive (MICs ≥64 mg/L) non-quinolones. Common to fluoroquinolones and the substances studied was their aromatic bicyclic structure. The methylxanthines caffeine (CNS-stimulant) and theophylline (bronchodilator), the quinines chloroquine and primaquine (antimalarials), chinosol (disinfectant), phenprocoumin (anticoagulant), allopurinol (antiurolytic), menadion (vitamin K), as well as quercetin and apigenin (natural dietary flavonoids) were studied. Susceptibilities to nalidixic acid served as an indicator for fluoroquinolone resistance in *E. coli* exposed to these agents for seven days in the absence of any antibiotic; amoxicillin served as a negative control. Increases in nalidixic acid MICs (8 to ≥32 mg/L) at day 7 but reversion to pre-exposure levels (≤2 mg/L), i.e. adaptive resistance, were observed with: theophylline, coffeine, allopurinol, marcumar, menadione, quercetin, apigenin and chinosol. In contrast, stable and thus mutational nalidixic acid resistance was observed with chloroquine and primaquine. Sequence analysis of the quinolone resistance determining region (QRDR) revealed that quinine-exposed mutants had a single mutation resulting in an amino acid exchange from aspartate to glycin at codon 87 of the *gyrA* gene. Pre- and post-exposure MICs of ciprofloxacin for these mutants rose from ≤0.015 mg/L to 0.25 mg/L. Reserpine had no effect on the MICs indicating that no major facilitator-type efflux pumps were involved in the expression of quinolone resistance [382]. These findings may help to explain why 4.8% of patients living in remote villages in South America carried ciprofloxacin-resistant *E. coli* with QRDR mutations although quinolones were locally not available. However, chloroquine has been used extensively in association with a high prevalence of chloroquine resistance in *Plasmodium vivax*. Antibacterial agents were not found in drinking water, but chloroquine was demonstrated to be present [383]. Also, high resistance rates in both *Plasmodium spp.* [384, 385] and bacterial pathogens in Asia or elsewhere [386] may be associated with chloroquine therapy of malaria. Furthermore, adaptive responses to natural dietary flavonoids like quercetin and apigenin – both known to inhibit DNA gyrase [387, 388] – may lead to or enhance fluoroquinolone resistance development.

Therefore, combinations between antibiotics with structurally unrelated non-antibiotics should not only be considered from a pharmacological but also from a microbiological perspective.

A media specific vancomycin resistance mechanism has been described in MRSA. Multiple peptide

resistance factor (MprF)-mediated vancomycin resistance developed in physiological (i.e. RPMI) but not in bacteriological- (i.e. CA-MHB) media [101]. MprF renders *S. aureus* resistant to a wide range of cationic antimicrobial peptides [389-392]. Both, cationic antimicrobial peptides and daptomycin share some structural homologies related to their calcium-mediated ionization and membrane insertion, as well as peptide content [393-395]. MprF-mediated daptomycin resistance results in cross-resistance to vancomycin, and friulimycin [392]. Thus, cationic antimicrobial peptides trigger development of resistances to cyclic glycopeptides and lipopeptides, and vice versa, cyclic glycopeptides and lipopeptides decrease activities of cationic antimicrobial peptides [392]. Using a zebrafish infection model it was shown that innate immune responses could not control infection with a daptomycin-resistant *S. aureus* whereas the virulence of its daptomycin susceptible counterpart was attenuated. The immune evasion of the daptomycin-resistant strains was due to cross-resistance with cationic antimicrobial peptides [396]. This finding is well in agreement with observations that human serum induced daptomycin tolerance [397], and that cationic antimicrobial peptides selected MRSA strains with diminished daptomycin susceptibilities from patients who have never been treated with daptomycin [398-400]. Overall the data support the notion that evolution of antibiotic resistance should not only be evaluated under routine *in vitro* but also under clinically relevant conditions.

Several chemicals were found to affect *mar* (multiple antibiotic resistance) expression *in vitro.* Alterations of *mar* locus expression result in resistance not only to a variety of antibiotics but also to organic solvents, oxidative stress agents and disinfectants. The *mar* locus consists of the *marC*- and *marRAB* transcriptional units encoding an inner membrane protein and the *mar* repressor MarR, the activator MarA, and a small protein MarB. Among the >60 genes controlled by the *mar* operon is also *tolC* encoding for AcrAB efflux pump complex thus contributing significantly to the *mar* phenotype. The AcrAB efflux pump complex is characterized by a broad substrate specificity extruding nalidixic acid, fluoroquinolones, tetracycline, chloramphenicol, aminopenicillins, rifampin, dyes, and disinfectants. Apart from chloramphenicol and tetracycline, sodium salicylate, 2,3-dihydroxybenzoate and anthranilate (two metabolic intermediates of enterobactin and tryptophan biosynthesis), as well as plumbagin and high concentrations of paraquat (oxidative stress agents), 2,4-dinitrophenol, menadione, benzoate (a common food preservative), 4-hyrdoxybenzoate, acetaminophen, and the so-called non-specific biocides like triclosan induced *marRAB*. While sodium salicylate and substances containing phenolic rings directly bound to MarR, the other chemicals may likely induce MarR by indirect mechanisms [401-407].

Phenolic compounds like 4-hydroxyphenylacetic acid (4-HPA) are found in food like olive oil or beer and in human body fluids like saliva and are degraded by bacteria of the oral cavity or intestinal tract [408-410]. Bacterial genes involved in the degradation of 4-HPA are located upstream of the aminoglycoside phosphotransferase gene *aph(3')-IIb* and are activated by HPA [411, 412]. HPA-activated expression of *aph(3')-IIb* resulted not only in resistance to kanamycin, neomycin, butirocin, and seldomycin [411] but also to ampicillin, erythromycin, tetracycline and fluoroquinolones [412, 413]). This multi-drug-resistant *E. coli* strain harboured the R-plasmid pHPA coding for the quinolone efflux pump QepA [412, 413]. The *aph(3')-IIb* gene was located in a *P. aeruginosa* isolate on a mosaic gene providing resistance to $\geq$13 antibiotics including aminoglycosides, ß-lactams and quinolones [414-416]. Phenotypic aminoglycoside resistance was also induced by benzoic acid used in the preservation of food and beverages [417]. Thus, the almost omnipresent phenolic compound HPA is a potent inducer of multi-drug-resistance. Furthermore, a variety of spices like capsaicinoids extracted from cayenne pepper, capsanthin extracted from paprika, tarragon used as a flavouring agent, or various phenols extracted from dill induced the *mar* operon [418]. Unspecific selection of adaptive or mutational antibiotic resistance by non-antibiotics may also explain the emergence of antibiotic- resistant pathogens in infected but untreated control animals. Such findings may have been made coincidentally as usually the emergence of resistance is evaluated in infected and treated animals only.

Data summarized in the preceding paragraph have shown that substances like aminoacids or naturally occurring flavonoids like quercetin and apigenin trigger resistance development either by acting as inducers/de-repressors or selectors. Thus, it is not surprising that ß-lactamase induction was detected in kidney homogenates of animals infected with *S. aureus* but untreated, and in untreated granuloma pouches infected with *P. mirabilis* [359, 360]. Glycopeptide resistances have been selected in MRSA infection models [419-421], fluoroquinolone resistances in a rat model of *P. aeruginosa* pneumonia [422], and macrolide resistances in mycobacterial infection models [423, 424] in untreated animals. Furthermore, emergence of vancomycin- [425] or daptomycin-resistant isolates has been reported among daptomycin- or vancomycin-naïve patients [398-400, 426, 427], multi-drug resistance has been observed in treatment-naïve tuberculosis patients [428] and metronidazole-, clarithromycin-, levofloxacin-, amoxicillin-, rifampicin- and tetracycline-resistances have been reported in treatment-naïve *H. pylori*-infected patients [429-431]. The emergence of antibiotic resistance in treatment-naïve patients provides supportive evidence but no proof for an unspecific selection of resistance as this phenomenon could in principle also be correlated with an uncontrolled consumption of antibiotics for the treatment of common infections in the general population.

The common denominator for the phenomena described above is the presence of subtle structural similarities between the stressor and the antibiotic against which resistance has been selected although the antibiotic and the stressor had to be assigned to totally different classes of substances. These findings support the conclusion that subtle structural similarities between antibiotics and non-antibiotics are completely sufficient to trigger resistance development. Furthermore, these findings suggest that environmental substances, consumer products, food, and body fluid constituents may possibly reduce the clinical efficacy of antibiotics; this being a totally neglected effect in the context of resistance development.

6. Induction of antibiotic resistance by structurally unrelated antibiotics

Inducible vancomycin resistance in enterococci is encoded by the VanRS system. The expression of *vanA* gene cluster is controlled by a two-component signal transduction system encoded by *vanS* and *vanR* genes. By screening a panel of more than 6,000 synthetic structurally diverse compounds it was found that not only did the three tested glycopeptides vancomycin, avoparcin and ristotecin induce vancomycin resistance in *E. faecium* but also several non-glycopeptides. Amongst the non-glycopeptides with an enterococcal VanRS induction potential were cell wall-active compounds differing in structure and mode of action from vancomycin such as the phosphoglycolipid moenomycin, cyclic polypeptides such as bacitracin, the lipopeptide polymyxin, the lipoglycodepsipeptide ramoplanin, robenidine, a 344-Da amino guanidine used as an anti-coccidial agent, and moxidectin, a macrocyclic lactone [432]. However, published data on this topic are in part contradictory. While some authors concluded that bacitracin is an inducer [432-435], others found that the opposite was true [436-438]. Furthermore, one group [439] described that ramoplanin was an inducer, while another one [437] concluded that it was not. It was also found that the bis-biguanide antiseptic chlorhexidine induced expression of *vanA*-type vancomycin resistance genes [440]. Chlorhexidine also induced genes associated with daptomycin resistance probably due to the synthesis of peptidoglycan precursors that terminate in D-Ala–D-Lac, but without being cross-linked due to changes in transpeptidase expression [440]. These in part contradictory findings beg the question of whether reactions either preceding or following transglycosylation play pivotal roles in the induction of vancomycin resistance of the vanA type. The following findings suggest an answer. While amphomycin, a lipocyclodecapeptide, moenomycin, a phosphoglycolipid also known as nonribosomal peptide antibiotic, and penicillin G inhibiting late stages of peptidoglycan synthesis induced vancomycin resistance, D-cycloserine and fosfomycin inhibiting early steps of peptidoglycan synthesis did not induce vancomycin resistance [432]. Further information is provided by the finding that

ß-lactamase expression is not only induced by ß-lactams and vancomycin, moenomycin, and ramoplanin in *E. coli* harbouring the ß-lactamase gene from *Citrobacter freundii*, but also by D-cycloserine and fosfomycin. In addition, ß-lactamase production was induced by these antibiotics in a mutant carrying a mutation in the *murG* gene converting the cell wall intermediate Lipid I to Lipid II. This therefore provided proof that induction of ß-lactamase production by non ß-lactams is a direct consequence of cell wall inhibition resulting in an accumulation of cell wall building blocks [441]. Regardless of the unresolved question of whether reactions either preceding or following transglycosylation play pivotal roles in induction of vancomycin resistance of the vanA type, data suggest that the induction signal for VanA resistance is not necessarily a structural glycopeptide feature. It may rather be sufficient for the effector ligand to be an intermediate in cell wall biosynthesis, accumulating due to inhibition of cell wall synthesis by structurally unrelated antibiotics. Genomic and metabolomic analysis may not only help to identify drug targets but also to characterize effectors mediating development of antibiotic resistance.

Phenotypic, genomic and metabolomic analysis revealed that production of DNA-damaging reactive oxygen species (ROS) thus inducing SOS response is a common trigger of bacterial cell death [442, 443]. Although this theory was initially discussed controversially, several studies have proven that a variety of antibiotics trigger ROS formation accounting for their antibacterial effects. Fluoroquinolones, aminoglycosides and ß-lactams induce metabolic shifts resulting in the accumulation of ROS [271, 444-455]. ROS-mediated DNA damage triggers the SOS response. Beta-lactams preferentially binding to PBP 3 [455-462], aminoglycosides [460, 462-465], and fluoroquinolones [454, 460, 461, 465-472], but also to tetracycline [460, 462] and chloramphenicol [462, 465], trimethoprim [473, 474] as well as co-trimoxazole [461] and nitrofurantoin [461], metronidazole [475], erythromycin [465], and polymyxin [476] induced SOS responses. Furthermore, zidovidine [473, 477, 478], 5-fluorouracil [479] and several dideoxynucleosides [478] with anti-human immunodeficiency virus activity induced SOS response in *E. coli*. The SOS response comprises the de-repression of more than 20 genes under the direct and indirect transcriptional control of the LexA repressor. As the SOS response promotes survival to fluoroquinolones it can be expected that non-quinolones inducing SOS response should induce parallel quinolone resistance; vice versa, quinolones should select for multiple antibiotic resistance. MIC determinations in 24 Gram-negative rods revealed that in addition to resistance selection to itself pefloxacin and ciprofloxacin selected for ß-lactam and aminoglycoside resistance in 11 and 8 strains, respectively, ceftazidime selected in turn for quinolone and aminoglycoside resistance in six strains, and amikacin selected for quinolone and ß-lactam resistance in four strains [480]. A study with 8 non-fermenters and 10 *Enterobacteriaceae* revealed that quinolones selected for ß-lactam resistance in 26% and ß-lactams selected for quinolone resistance in 7% of the strains studied [481]. Others generated similar results. One of five strains of *K. pneumoniae* selected with nalidixic acid and ciprofloxacin, respectively, and three of four strains selected with norfloxacin were cross resistant to quinolones and ß-lactams, and four of four strains selected by cefotaxime were quinolone- and amikacin cross-resistant. Additional cross-resistant mutants of *P. stuartii, E. cloacae, S. marcescens* and *P. aeruginosa* were selected by either of these agents [482]. The genetic background has not been examined in any of these studies. These examples demonstrate that cross-resistances did not emerge according to the all or nothing principle, and hence a representative number of strains should be tested. Cross-resistance developed in a patient from whom a pre-therapy *S. marcescens* isolate susceptible to amikacin, gentamicin, co-trimoxazole, mezlocillin, cefoxitin and ciprofloxacin but resistant to ampicillin and cefazolin was isolated. Initial therapy with ticarcillin and tobramycin was switched to cefazolin and gentamycin. Resistance to ß-lactams, aminoglycosides and ciprofloxacin developed during therapy [483]. Development of quinolone resistance in this patient is remarkable in so far as the patient was treated during the pre-quinolone era in the USA. Clearly, these phenotypic descriptions of cross resistances between structurally unrelated antibiotics do not provide direct proof for a SOS-driven selection of cross resistances but

it can be seen as a supportive evidence. Direct proof for a SOS response-induced resistance development was provided by three *in vitro* studies paralleled by studies in experimental animals and in a clinical setting. *In vitro* exposure of *E. coli* to ciprofloxacin and ziduvidine was found to select for rifampicin, minocycline, and fosfomycin resistance as a direct consequence of SOS induction. Both agents triggered resistance development *in vivo* in a rabbit model of intestinal infection with enteropathogenic *E. coli* [478]. Metronidazole-induced SOS response was observed in a patient suffering from a *P. aeruginosa* and "anaerobes" infection. The patient was treated with ceftazidime and metronidazole, both known to trigger the SOS response. The pre-therapy *P. aeruginosa* isolate was, in contrast to the post-therapy isolate, susceptible to ceftazidime. An *in vitro* analysis of this isolate revealed that ceftazidime did not induce the SOS response in this strain but metronidazole did paralleled by increased ß-lactamase expression [475]. The LexA repressor was found to be essential for resistance development to ciprofloxacin or rifampicin in an *E. coli* thigh infection model in neutropenic mice [475] thus demonstrating that resistance development was dependent on the SOS system.

One of the multiple changes in bacteria triggered by the SOS response is the increased expression of error-prone non-essential DNA polymerases Pol II, Pol IV, and Pol V on LexA-cleavage-mediated derepression of their respective genes (*polB, dinB*, and *umuDC*) resulting in increased mutation rates [484-486]. Adaptive responses due to growth in hostile environments are regulated by the SOS response and thus paralleled by increased mutation rates [487]. For example, in *E. coli* ciprofloxacin increased mutant frequencies by four orders of magnitude [484, 488], in *P. aeruginosa* ceftazidime increased evolution of rifampicin resistance by one order of magnitude within 5 hours [388], in *E. coli* ciprofloxacin increased evolution of rifampicin resistance three-fold per generation [489], and peroxide increased resistance to aminoglycosides by 1.3- to 8.4-fold [271]. In *M. fortuitum* ciprofloxacin increased mutant frequencies by 72 to 120-fold [490]. These data prove that the antibacterial as well as the antiviral agents studied and peroxide induced expression of the SOS pathway were paralleled by an increased mutagenesis and an augmented evolution of antibiotic resistance.

Data summarized in this paragraph demonstrate that emergence of resistance is driven not only by structural homology of the agents and/or identical modes of action or resistance mechanisms but also by corresponding downstream effects triggered by structurally unrelated antibiotics with different targets. The common denominator in this case is the confluence of downstream effects triggered by diverse agents into a common signal transduction network leading in the end to antibiotic resistance. These data also imply that a monocausal correlation between antibiotic consumption and the development of resistance masks the multiple factors contributing to resistance development.

7. Conclusions

The data summarized above clearly indicate that both, the terminology and methods used should be standardized as otherwise results generated by using routine procedures vary widely resulting in inconsistent and even contradictory data. Conclusions drawn may be misleading because of inappropriate terminology. A standardization of methods used is in principle easily achievable and could follow the model of harmonizing MIC testing. More important but also more complex than such technicalities are the following concepts. Regulatory authorities request information about the frequency of selection of resistance either by exposing strains overnight to constant drug concentrations or by using *in vitro* pharmacodynamic models simulating fluctuating drug concentration profiles that mimic those achieved in infected patients [42]. The optional selection of methods implies that data thus generated should be comparable. However, mutant frequencies obtained with one method or another differed by up to eight orders of magnitude. The development of resistance appeared very unlikely if mutant frequencies were determined with the discrete endpoint method exposing bacteria to constant concentrations for 18 h whereas resistance development was predominant in PK simulation models or in experimental animals [46-50]. Discrepant findings were also obtained if bacteria were either grown in commercial media or in humanized media and human body fluids. Mutant frequencies were mostly low in the first

case but almost always high in the latter case [70-96, 491, 492]. These contradictory data can best be explained by adaptation to growth in an *ex/in vivo* environment as compared to routine *in vitro* conditions affecting antibacterial activities of antibiotics significantly irrespective of their chemical class or mode of action [262, 263, 267, 268, 274, 275, 282-287, 290-296]. Consequently, growth condition specific shifts in bacterial physiology and metabolism affecting resistance development should be taken into account. In addition, a few indicator strains per species with unknown clinical background are usually used assuming that the parameter mutant frequency is a species-specific- and universally applicable parameter rather than linked to various pathologies. However, mutant frequencies and percentage of mutator strains of various *E. coli* strains isolated from patients either suffering from urinary tract or abdominal infections or bacteraemia differed significantly. Uropathogenic strains had the highest and bacteraemic strains had the lowest mutant frequencies [493]. Therefore, pathophysiologically relevant conditions at the focus of infection should be considered and should be reflected in the experimental design. In addition, there was no correlation between mutant frequencies and clinically relevant antibiotic resistance [493]. Likewise, *in vitro* mutant frequencies for oxazolidinone and mecillinam resistance differed by five orders of magnitude but the emergence and frequency of resistance in clinical settings did not differ [494]. These findings and data quoted in the introductory remarks indicate that the parameter "mutant frequency" is a suboptimal predictor of the risk of developing resistance and that methods used to quantify the risk of emergence of resistance were and remain inappropriate. This also applies inversely for susceptibility testing [95].

Furthermore, regulatory authorities demand information about the mode(s) of action and mechanisms(s) of resistance based on the assumption that interactions of antibiotics with their target(s) would be identical under *in vitro* or *in vivo* conditions. However, this is not always the case. The expression of PBPs in particular is highly variable *in vitro* and *in vivo* such that essential PBPs targeted by a ß-lactam *in vitro* may not be expressed *in vivo*. Likewise, gyrase A and gyrase B are expressed *in vitro* during the exponential growth phase but only minimally and not all during the late exponential or stationary phase, respectively, corresponding to the growth status of bacteria at infectious foci. Thus, the target considered to be essential under routine *in vitro* conditions may become lost under pathophysiologically relevant *in vivo* conditions. Consequently, ß-lactams or fluoroquinolones may not just only interact with their well-known targets, i.e. PBPs or bacterial type II topoisomerases, but may interact with additional targets alternative to or downstream from these targets [324, 325, 326]. Analogues findings were generated for fatty acid synthesis inhibitors, isoniazid, tetracyclines, daptomycin, and dihydrofolate inhibitors [350-358]. The conditional or temporal essential nature of targets or resistance mechanisms therefore challenges the generally applied practice of extrapolating from *in vitro* to *in vivo* conditions on the assumption that growth in one or another environment would not affect target expression or resistance mechanisms, whereas the opposite is the case.

Moreover, the limitation to evaluating the extent of cross-resistance exclusively within the class, provided that an agent of an existing class is being developed [42], seems to be too simplistic. Numerous studies have demonstrated that subtle structural homologies between non-antibiotics and the antibacterial agents are sufficient to induce resistance development and to select for cross-resistance [361-366, 371-376, 382, 401-418]. However, structural homology *per se* is not always a driver of resistance development as demonstrated by the structural similarity between linezolid and rivaroxaban (an oral anticoagulant with a direct anti-Xa activity) sharing the central 5-S oxazolidinone structure, which is essential for linezolid's antibacterial activity. Nevertheless, neither rivaroxaban exhibited an antibacterial activity, nor did linezolid affect rivaroxaban's potential mitochondrial toxicity [495] Therefore, such triggers should be analysed on an individual drug-drug relationship. As a variety of non-antibiotics are antibacterially active and even reverse antibiotic resistances, whilst antibiotics affect eukaryotes due to identical modes of actions deriving from structural and functional

homologies of pro- and eukaryotic targets [496, 497], one should address the question of whether antibacterially inactive compounds with subtle structural homologies to antibiotics may elicit adaptive or mutational resistances or, vice versa, may cause adaptive susceptibility.

Furthermore, the emergence of resistance is not only driven by structural homology of the agents and/or identical modes of action or resistance mechanisms but also by downstream effects triggered by structurally unrelated antibiotics with different targets but ultimately leading to a common network of regulatory components. These signal transduction pathways like e.g. SOS response result in antibiotic resistance across various drug classes [432-441, 480-490]. Consequently, statements made in publications or in product monographs to the effect that cross-resistance between antibacterial agents of different classes does not generally exist because of different modes of action are misleading. Equally inadequate is the limitation imposed by the authorities requiring that the evaluation of cross resistance within the class be subject solely to the agent of an existing class being under development.

The question of whether resistance may emerge should thus not only be addressed from a target oriented mechanistic position but also from a physiological perspective. This should be linked to pathologies in humans and bacterial physiology and regulatory networks as well as from a biochemical perspective taking into account the structural homologies of antibiotics and environmental stressors as well as precursors of antibiotic biosynthesis and the agent itself. Simulation of pathophysiologically relevant growth conditions should therefore be attempted. This could make a significant contribution towards solving the problem of discrepant findings generated by using either routine *in vitro* growth conditions or approaches mimicking infectious sites. Assays performed under such conditions have a greater potential to generate clinically relevant data on either the emergence of resistance or the activity of novel agents against resistant bacteria that would be missed by experiments performed in conventional laboratory media under routine conditions. A word of caution should be expressed in this context. As reviewed previously [102, 115, 234, 498-500], pathogens growing in different habitats are not biologically equivalent and antibacterial activities may differ significantly in non-clinical model systems and in the clinical arena and hence findings generated in model systems may not be applicable in humans. It should therefore be ensured that the model used mimics crucial properties similar to those found in humans.

8. Perspective

The prediction of the risk of resistance development could probably be improved if not only mutant frequencies were quantified and mechanisms of resistance analysed but if additional aspects were likewise addressed. Apart from the use of an appropriate medium simulating growth conditions at the infectious site, the issue of whether bacteria might be cultured in the chemostat simulating *in vivo* growth rates, instead of in batch cultures [501] has not been pursued further since 1990s. The use of human organoids will perhaps show us a completely new way forward in the analysis of infectious diseases, their treatment and factors affecting emergence of resistance. Several approaches to model viral or bacterial infections in human organoids have been published [98, 502-516]. Regulatory mechanisms like sigma factors affect expression of resistance genes. Recently it has been shown that alternative sigma factors mediated antibiotic resistance, too [206, 264, 279, 517, 518]. Two component regulatory systems are known since long to increase antibiotic resistance. The elucidation of such regulatory networks could help explaining expression of resistance mechanisms or drug targets shaped by environmental stressors in the absence of antibiotics. In general, metabolic, communication and regulatory profiles and networks should be analysed in addition to a characterization of target interactions and resistance mechanisms to generate an integrated and holistic scheme for drug action and resistance mechanisms as well as resistance development.

So far most studies have assayed a single condition, e.g. exponential growth in a nutrient-rich medium, and have thus not differentiated between housekeeping genes and their expression

stabilities from those that are specific to a particular pathophysiologically relevant condition. Well-regulated gene control systems have been developed allowing titratable induction of gene expression over a rather broad range correlating well with the physiological expression levels of cellular proteins [319, 321]. Whether such procedures would allow condition-specific analysis of antibacterial activities and resistance development remains to be analysed.

Population biological processes should also be considered. Pathogens multiply at some infectious foci within the natural resident flora, but are studied *in vitro* in monocultures as if they would cause infections in otherwise sterile sites. It has been shown that selection for resistance was reduced or even reversed when resistant bacteria grew within mixed bacterial populations [519-521]. On the other hand, horizontal gene transfers could spread resistance within such populations. A different way of thinking is represented by the concept of collateral sensitivity occurring during evolution of antibiotic resistance. This concept describes the phenomenon that resistance to one antibiotic simultaneously confers increased susceptibility to another chemically unrelated agent [522-527]. However, evaluation of mechanisms resulting in collateral sensitivity is at an early stage and clinical utility of this trade-off is so far unclear [522, 527-529].

The use of methods other than those applied routinely could contribute to a better identification and prediction of antibiotic resistance. For example, teixobactin, a nonribosomal peptide antibiotic, was described as "a new antibiotic that kills pathogens without detectable resistance" [530]. No mutants of *S. aureus* or *M. tuberculosis* resistant to teixobactin could be elicited neither following incubation for 18 h nor serial passage of *S. aureus* over a period of 27 days. However, multistep experimental evolution revealed that resistance to Arg_{10}-teixobactin (an analogue in which the nonproteinogenic amino acid l-allo-enduracididine was substituted by arginine) in *S. aureus* emerged slowly over 45 days of selection [531]. Resistance to moenomycin, another nonribosomal peptide antibiotic with low mutant frequencies for resistance ranging from 10^{-9} to 10^{-10} within 18 h [532], increased rapidly and significantly during multistep exposure [531]. By using whole genome sequencing and structural biology it was demonstrated that genes involved in cell wall modulation, lipid biosynthesis and energy metabolism were involved in the evolution of teixobactin and moenomycin resistance [531]. Application of a genome-mining platform with 5,585 complete bacterial genomes spanning the entire domain of bacteria revealed that D-stereospecific peptidases represent a widespread and broad-spectrum mechanism of resistance to nonribosomal peptide antibiotics [533]. Known functions of D-stereospecific peptidases are cell wall assemblage or inactivation of ß-lactams, thus indicating that in principle these enzymes could mediate cross-resistances to antibiotics of drug classes other than nonribosomal peptide antibiotics. The integration of gene expression profiles, biochemical, physiological and structural data and additional data sets like resistance phenotypes and resistance mechanisms into statistical and machine learning methods [119, 534-539] provide tools for identification and prediction of antibiotic resistances, novel resistance mechanisms, cross resistances as well as environmental distribution of resistance genes. The practical applicability of such approaches in research and drug development must be proven.

Clearly, the microbiologist has to characterize basic parameters like target affinity, resistance mechanism(s), pharmacodynamics, etc. by using well-defined and standardized *in vitro* methods. Findings thus generated should be embedded into the complex and multifactorial world of *ex-/in vivo* models to prove if data thus generated may or may not translate into the clinical arena. Thus, the microbiologist should accomplish a twofold turn first turning from diversity of clinical settings to unity of *in vitro* experiments but then switching back from unity to diversity of *ex-/in vivo* models to check if the working hypothesis is still viable or ought to be modified [60, 102, 500]. Furthermore, antimicrobial screens performed under *in vivo*-like conditions may have the potential to identify agents that would be missed by screens performed in conventional laboratory media using standard methods. Another question worth discussing is if not only the methods used but also *in vitro* test algorithms that correlate better with clinical efficacy should replace the traditional parameter "mutant frequency".

CONFLICT OF INTEREST STATEMENT

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