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Evaluation of Reduced Susceptibility to Quaternary Ammonium Compounds and Bisbiguanides in Clinical Isolates and Laboratory-Generated Mutants of *Staphylococcus aureus*

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The MICs and minimum bactericidal concentrations (MBCs) for the biocides benzalkonium chloride and chlorhexidine were determined against 1,602 clinical isolates of *Staphylococcus aureus*. Both compounds showed unimodal MIC and MBC distributions (2 and 4 or 8 mg/liter, respectively) with no apparent subpopulation with reduced susceptibility. To investigate further, all isolates were screened for *qac* genes, and 39 of these also had the promoter region of the NorA multidrug-resistant (MDR) efflux pump sequenced. The presence of *qacA*, *qacB*, *qacC*, and *qacG* genes increased the mode MIC, but not MBC, to benzalkonium chloride, while only *qacA* and *qacB* increased the chlorhexidine mode MIC. Isolates with a wild-type *norA* promoter or mutations in the *norA* promoter had similar biocide MIC distributions; notably, not all clinical isolates with *norA* mutations were resistant to fluoroquinolones. *In vitro* efflux mutants could be readily selected with ethidium bromide and acriflavine. Multiple passages were necessary to select mutants with biocides, but these mutants showed phenotypes comparable to those of mutants selected by dyes. All mutants showed changes in the promoter region of *norA*, but these were distinct from this region of the clinical isolates. Still, none of the *in vitro* mutants displayed fitness defects in a killing assay in *Galleria mellonella* larvae. In conclusion, our data provide an in-depth comparative overview on efflux in *S. aureus* mutants and clinical isolates, showing also that plasmid-encoded efflux pumps did not affect bactericidal activity of biocides. In addition, current *in vitro* tests appear not to be suitable for predicting levels of resistance that are clinically relevant.

Antimicrobial compounds, which include antibiotics, are almost exclusively intended for direct human or animal use. Biocides, on the other hand, have a much wider range of application, including disinfectants, preservatives, pest control agents, and other products (1). Despite the continuous and widespread use of biocides, detailed information on possible resistance mechanisms in clinical isolates is still lacking (2–4).

Active efflux is one of the main mechanisms of resistance to antibiotics and biocides. All bacteria have efflux systems which share a broad substrate specificity, including cationic biocide compounds. These transporters are known as multidrug-resistant (MDR) efflux pumps and belong to distinct transporter families (5). NorA is the chromosomally encoded MDR efflux pump in *Staphylococcus aureus* (6), with norfloxacin (NOR) and ciprofloxacin (CIP) being the most clinically relevant substrates (7). NorA also confers resistance to a broad range of other compounds, including lipophilic, monocationic compounds (ethidium bromide [EB], cetrimide, benzalkonium chloride [BZC], and acriflavine [AF]) (8, 9). NorA-induced resistance typically arises from increased expression of the efflux gene due to mutations in the *norA* promoter region (10–12).

Of the plasmid-encoded MDR efflux pumps, 6 different *qac* genes have been described in *S. aureus* (*qacA*, *qacB*, *qacC*, *qacG*, *qacH*, and *qacJ*) (13–18). Among these, the most frequently encountered pump is the QacA protein, which mediates resistance to a number of classes of antimicrobial organic cations, including intercalating dyes (e.g., ethidium bromide and acriflavine) and quaternary ammonium compounds (QAC) (13). Next in frequency of detection is the *qacC* gene, which encodes a small membrane efflux protein of the SMR family and has a more restricted substrate profile (14). QacB protein, similar to QacA except for seven nucleotide polymorphisms, confers reduced susceptibility to diamidines and biguanides (15). Other plasmid-located *qac* genes, *qacG*, *qacH*, and *qacJ*, have been identified in food-borne and veterinary isolates of *S. aureus* (16–18). Being located on plasmids, the *qac* genes can be transferred horizontally, and strains carrying *qac* genes have been isolated worldwide (19).

It has been suggested that widespread use of biocides affects the prevalence of antibiotic-resistant microorganisms (2, 20, 21). The increased number of formulations/products containing biocides, often at low concentration, raises concerns over the risk of selection of biocide-resistant strains (2, 20, 21). By mechanisms of coresistance and cross-resistance, such strains also could become

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antibiotic resistant and possibly represent a problem of clinical relevance (2, 20, 21). In this context, recent guidelines and policies aim to introduce tests for risk assessment for biocide resistance development. Still, no standardized methodology is available to run such tests. In the case of the biocide triclosan, we recently demonstrated the low predictive value of the *in vitro* test to predict clinically relevant biocide resistance (22). The aim of the present work is to provide insight into the factors to be taken into account for a risk analysis of resistance to the widely used quaternary ammonium compounds and bisbiguanides. For this scope, we performed a comparative molecular and phenotypic characterization of the susceptibility to benzalkonium chloride and chlorhexidine (CHX) in the clinically relevant model organism *S. aureus*. This work is part of the multicenter BIOHYPO project, which aims to evaluate the impact of biocide use in the food chain on antimicrobial drug resistance of clinical relevance in enterobacteria, Gram-negative nonfermenters, staphylococci, enterococci, lactic acid bacteria, and fungi (2, 22–27). In this context, the detailed characterization of biocide susceptibility phenotypes and genotypes is the first step of correlation of these data with antimicrobial resistance profiles.

MATERIALS AND METHODS

Bacterial strains. A collection of 1,602 *S. aureus* strains collected in 2002 to 2003 from different geographical origins, representing both hospital- and community-acquired infections and hosted at the strain collection of Quotient Bioresearch (Fordham, United Kingdom), was investigated. The same strain collection had previously been screened for susceptibility to the biocide triclosan (22). *S. aureus* strains used for *in vitro* mutant selection included the biocide reference strain ATCC 6538, the standard laboratory strain RN4220, the classical reference strain for antimicrobial susceptibility testing, ATCC 2593, and three methicillin-resistant *S. aureus* (MRSA) strains with sequenced genomes, MW2, COL, and Mu50, of which the latter two harbor plasmids containing the quaternary ammonium compound resistance gene (*qacA*) (22).

Chemical agents. Compounds used were ethidium bromide (EB; 10 mg/ml; Fluka, Steinheim, Germany), ciprofloxacin (CIP; 2 mg/ml; Bayer, Leverkusen, Germany), norfloxacin (NOR; 50 mg/ml in acetic acid; N9890; Sigma, Steinheim, Germany), benzalkonium chloride (BZC; 100 mg/ml in water; B6295; Sigma), chlorhexidine digluconate (CHX; 100 mg/ml in water; C9394; Sigma), and acriflavine (AF; 100 mg/ml in dimethylsulfoxide [DMSO]; A8126; Sigma).

Susceptibility testing. MICs were determined using the broth microdilution method (28). Minimum bactericidal concentrations (MBCs) were determined by subculturing 10 μl from each well without visible bacterial growth on Mueller-Hinton agar plates. After 24 h of incubation at 37°C, the dilution yielding three colonies or fewer was scored as the MBC, as described by the CLSI for starting inocula of 1×10^5 CFU/ml (29). Reference strains were included in all 105 MIC and MBC determinations, and we confirmed the reliability of the susceptibility tests for the biocidal compounds by evidencing deviations from the mean results of only one dilution.

MLST analysis. Multilocus sequence typing (MLST) was performed on a group of 91 clinical isolates carrying *qac* determinants as described previously (30). The allelic number and STs were assigned using the *S. aureus* MLST database (http://saureus.mlst.net), while the clustering of related STs, defined as clonal complexes (CCs), was analyzed with the BURST algorithm (http://eburst.mlst.net). New alleles and STs have been submitted to the *S. aureus* MLST database.

Activity testing. Benzalkonium chloride and chlorhexidine activity testing against the reference *S. aureus* strain and selected isolates were performed by following EN 1276 (31). Briefly, 1 ml of a test suspension of microorganisms at a concentration between 1.5×10^8 and 5×10^8 CFU/ml was mixed with albumin from the bovine serum Cohn V fraction (A2153; Sigma) at a concentration of 0.03 g/liter. After 2 min, 8 ml of the test product solution was added and mixed. Test product solutions were obtained by diluting benzalkonium chloride (B6295; Sigma) and chlorhexidine digluconate (C9394; Sigma) in hard water (119 mg/liter $MgCl_2$, 277 mg/liter $CaCl_2$, and 280 mg/liter $NaHCO_3$). The mixture was maintained at 20°C (±1°C) in the test tube for 5 min (±10 s). After this contact time, a 1-ml aliquot from the test tube was transferred to a tube containing 8 ml of the neutralizer (3 g/liter lecithin, 30 g/liter polysorbate-80, 5 g/liter sodium thiosulfate, 1 g/liter L-histidine, and 30 g/liter saponin in diluent) and 1 ml of water. After 5 min of neutralization, dilutions of the neutralized suspension were performed in diluent (0.14 mM NaCl plus 0.1% tryptone). One ml of each dilution, ranging from the neutralized suspension to a 10^{-3} dilution, was cultured in duplicate on tryptic soy agar (TSA; Liofilchem, Roseto degli Abruzzi, Italy) using the pour plate technique. Plates were incubated at 37°C (±1°C) for 48 h. Calculations of log reductions and expression of results followed the provisions of European Standard (EN) methods (31).

Selection of mutants. For selection of single-exposure mutants, strains were grown overnight and approximately 10^{11} CFU was plated on TSA containing either ethidium bromide (64 mg/liter), acriflavine (64 mg/liter), benzalkonium chloride (16 mg/liter), or chlorhexidine (8 mg/liter). Plates were examined for growth after 48 h. Multiple-exposure mutants were produced by five serial passages on plates containing increasing concentrations from 1 to 16 mg/liter of benzalkonium chloride and 0.5 to 8 mg/liter of chlorhexidine. Single colonies obtained by both methods were randomly selected for further analyses.

Qualitative real-time PCR amplification of *qac* genes. Genomic DNA was extracted using the High Pure PCR template preparation kit (Roche Diagnostics, Germany). Separate fragments of DNA internal to each of four *qac* genes were amplified by real-time PCR using the primers described in Table S1 in the supplemental material and SYBR green I dye (Roche Diagnostics, Germany). Two TaqMan probes with two different fluorophores at the 5′ end and a minor groove binder (MGB) at the 3′ end (Applied Biosystems, United Kingdom) were used in order to distinguish between *qacA* and *qacB*. Qualitative real-time PCRs were performed in a LightCycler 480 system (Roche Diagnostics, Germany). The two *qacB*-positive strains were confirmed by sequencing with the Sanger method (BMR Genomics, University of Padova, Italy).

Screening of the *norA* promoter region. A 457-bp region upstream of *norA* in a subset of 49 clinical isolates was amplified using primers NorAp_F and NorAp_R (see Table S1 in the supplemental material) and was designed on the basis of the *S. aureus* MW2 chromosome using standard procedures. Strains were selected to be representative of the full range of ethidium bromide MICs. PCR fragments were submitted for sequencing to BMR Genomics (University of Padova, Italy). The nucleotide positions of intergenic regions are numbered backwards, starting at the *norA* start codon (NC_003923, position 739144).

Whole-genome sequencing. Four clinical isolates with benzalkonium chloride MBCs of ≥32 mg/liter and carrying no *qac* determinants were sequenced. Whole-genome sequence data were analyzed as described in Ciusa et al. (22).

***Galleria mellonella* infection model.** As described before, final-instar larvae of *G. mellonella* (Allevamento Cirà, Como, Italy) were stored in wood shavings in the dark at 15°C and used within a week after shipment. Overnight cultures of *S. aureus* were resuspended in phosphate-buffered saline (PBS). *G. mellonella* was injected with 10^5 CFU/larva, directly into the hemocoel via the last left proleg, using a Hamilton syringe (26, 32). Larvae were incubated at 37°C in petri dishes, and survival was evaluated for 6 days. Each experimental group contained 16 larvae of appropriate weight (0.3 to 0.5 g). All experiments included an equal number of larvae injected with PBS and noninjected larvae. At least three independent assays were performed for all *G. mellonella* killing experiments. Survival curves were estimated by the Kaplan-Meier method, and differences in survival were calculated using the log-rank test (STATA 6 software).

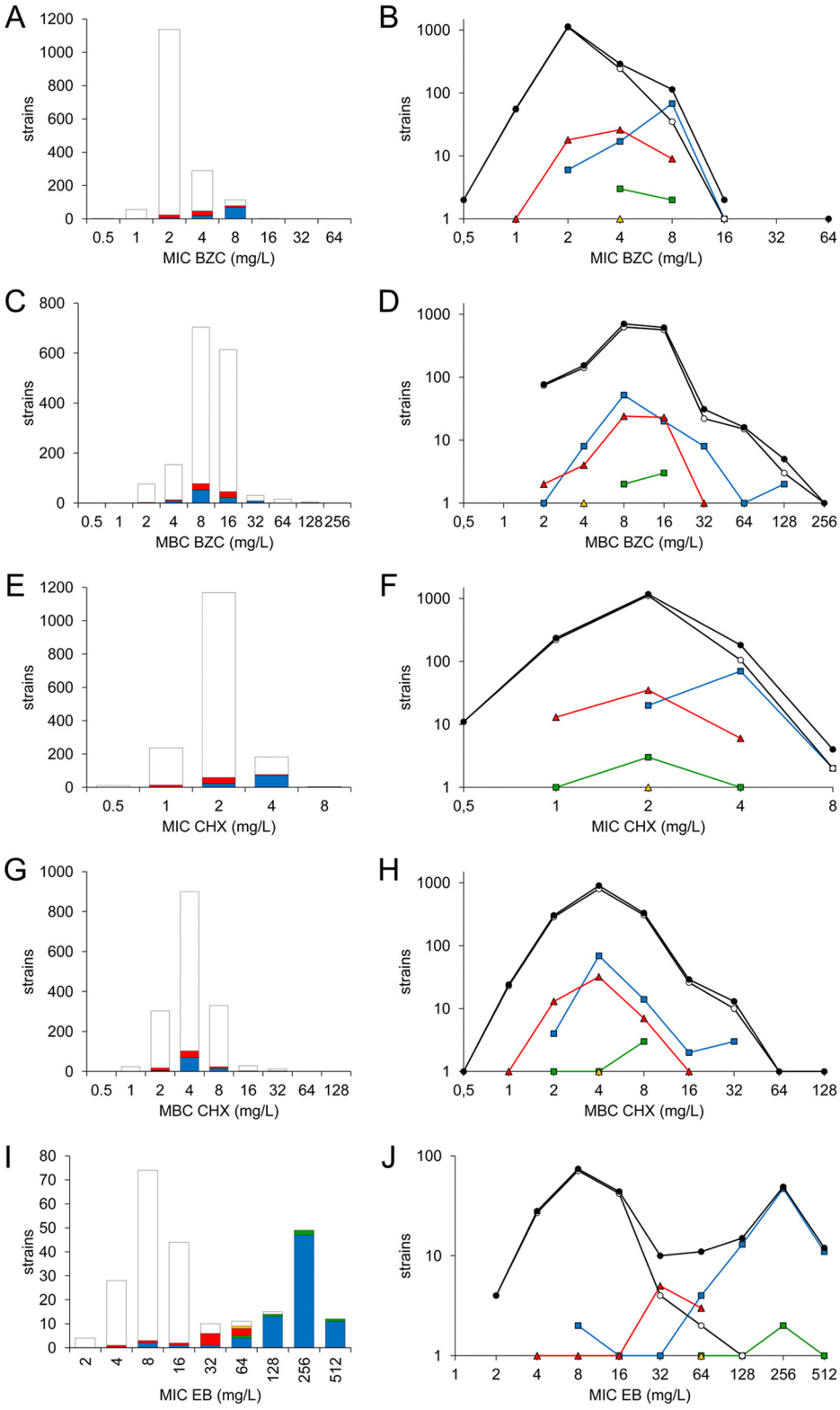

FIG 1 Phenotypic biocide susceptibility profiles of 1,602 *S. aureus* clinical isolates with genotype indicated. MIC and MBC distributions for benzalkonium chloride (A and C) and chlorhexidine (E and G) are shown. Molecular characterization of strains is plotted in a color scale, where white stands for the absence of *qacA*, *qacB*, *qacC*, *qacJ*, and *qacG*. The presence of *qac* determinants is shown in blue for *qacA*, in green for *qacB*, in red for *qacC*, and in yellow for *qacG*. (B, D, F, and H) Distribution of *qac* determinants in a log-scale plot where the color scheme is the same, with the exception of open circles that stand for the absence of *qac* genes and black filled circles that represent the total number of strains analyzed. In the case of ethidium bromide (I and J), phenotypic and genotypic data refer to a subgroup of 245 clinical isolates.

TABLE 1 Testing of chlorhexidine activity on *S. aureus* strains by following CLSI and EN 1276 guidelines

Strain	MIC (mg/liter)	MBC (mg/liter)	EN 1276 (log reduction, CFU/ml) guideline at[a]: 10 mg/liter	80 mg/liter	300 mg/liter	Note
ATCC 6538	2	4	<0.68	2.70	4.16	Wild type
QBR102278-1191	2	4	<1.27	4.84	>5.64	*qacG* positive
QBR102278-1387	2	4	<1.14	5.36	>5.51	Mutated *norA* promoter
QBR102278-1503	2	4	<1.03	2.16	3.67	*qacA* positive
QBR102278-2092	2	8	<1.30	5.36	>5.67	*qacC* positive

[a] Values report the logarithmic reduction (log *R*) of bacterial counts within 5 min of contact time and subsequent neutralization (the product is considered active if log $R > 5$).

Statistical analysis. Fisher's exact test was applied to contingency tables in order to determine if there were nonrandom associations between two categorical variables. Spearman's correlation coefficient, here denoted by ρ, measures the nonlinear statistical dependence between two monotonically dependent samples. The statistical tests were implemented using Matlab (version 2010b; MathWorks, Natick, MA).

Nucleotide sequence accession numbers. Sequences of the *norA* promoter regions of 13 clinical isolates (accession numbers JQ744024 to JQ744036) and 24 laboratory mutants (accession numbers JQ744037 to JQ744060) were deposited in GenBank.

RESULTS

Susceptibility of clinical isolates to biocides. MIC and MBC data for benzalkonium chloride and chlorhexidine were obtained from a series of 1,602 clinical isolates of *S. aureus* previously characterized for their profiles of susceptibility to triclosan (22). Susceptibility data are shown in Fig. 1. Both biocides produced a mode MIC of 2 mg/liter, with benzalkonium chloride having a mode MBC of 8 mg/liter and chlorhexidine a mode MBC of 4 mg/liter. The MIC or MBC distributions were unimodal, without any obvious subpopulation with reduced susceptibility. Only MIC data for benzalkonium chloride showed the presence of some isolates which could be considered non-wild type (benzalkonium chloride MIC of >4 mg/liter). The analyses of biocide activity according to the EN 1276 norm were performed on four clinical isolates, each carrying either a *norA* promoter mutation or a *qacA*, *qacC*, or *qacG* determinant (see below). Data indicate that chlorhexidine is not less active on strains QBR102278-1191 (*qacG*), QBR102278-1387 (*norA* promoter mutation), and QBR102278-2092 (*qacC*), and it is not significantly less active on strain QBR102278-1503 (*qacA*) (Table 1). Benzalkonium chloride was not less active against the four isolates tested (Table 2). Out of the 65 strains with low susceptibility to benzalkonium chloride (MIC of >4 mg/liter), only six had been found previously to show reduced susceptibility to triclosan (MBC of >4 mg/liter) (22).

Molecular characterization of clinical strains. For a more detailed analysis of the genotypes related to susceptibility to cationic compounds, the entire collection was analyzed for the presence of plasmid-encoded efflux pumps. Among the 1,602 strains, 92 (5.7%) were positive for *qacA*, 5 (0.3%) for *qacB*, 54 (3.4%) for *qacC*, and 1 for *qacG*. No *qacJ*-positive strains were found. In two strains, the *qacA* and *qacC* genes were detected concomitantly. When analyzing the presence of *qac* determinants and related phenotypes, data clearly showed that the benzalkonium chloride mode MIC was increased two dilutions by *qacA* and one dilution by the presence of the other *qac* determinants (Fig. 1B). In other words, most strains harboring a *qacA* determinant have a benzalkonium chloride MIC higher by two dilutions than wild-type staphylococci, while other *qac* genes determine an increase in MIC of most strains of only one dilution. The relationship between the presence of *qac* genes and a benzalkonium chloride MIC of >4 mg/liter is statistically significant ($P < 0.001$). It is noteworthy that two out of three strains with the highest MIC to benzalkonium chloride (>8 mg/liter) were *qac* negative. The presence of the four *qac* genes did not influence the benzalkonium chloride mode MBC (Fig. 1D). In the case of chlorhexidine, only the presence of *qacA* increases the mode MIC values of clinical isolates by one dilution (Fig. 1F). As for benzalkonium chloride, clinical strains with low susceptibility to chlorhexidine (MIC of >2 mg/liter) have a strong relationship with the presence of *qacA* determinant ($P < 0.001$), and MBC values were not affected by the presence of *qac* determinants (Fig. 1H). Correlation between increased MIC values for benzalkonium chloride and for chlorhexidine is statistically significant ($P < 0.001$), as is correlation between raised MBCs (benzalkonium chloride MBC of >16 mg/liter and chlorhexidine MBC of >8 mg/liter) ($P < 0.001$). Since there was no correlation between *qac* genes and MBCs (Fig. 1D and H), we have sequenced the whole genome of four *qac*-negative strains with benzalkonium chloride MBCs of ≥32 mg/liter but found no consistent changes in promoter regions of other efflux pumps (see Table S2 in the supplemental material).

When plotting ethidium bromide MIC values assayed in a subgroup of 245 clinical isolates, two clearly separated populations of strains became evident (Fig. 1I), which was quite distinct from the unimodal distribution observed for benzalkonium chloride or

TABLE 2 Testing of benzalkonium chloride activity on *S. aureus* strains by following CLSI and EN 1276 guidelines

Strain	MIC (mg/liter)	MBC (mg/liter)	EN 1276 (log reduction CFU/ml) guideline at[a]: 10 mg/liter	100 mg/liter	500 mg/liter	Note
ATCC 6538	2	8	<1.11	>5.48	>5.48	Wild type
QBR102278-1191	4	4	<1.25	>5.52	>5.52	*qacG* positive
QBR102278-1387	4	16	<1.24	>5.61	>5.61	Mutated *norA* promoter
QBR102278-1503	4	4	<1.16	>5.53	>5.53	*qacA* positive
QBR102278-2092	8	16	<1.17	>5.54	>5.54	*qacC* positive

[a] Values report the logarithmic reduction of bacterial counts within 5 min of contact time and subsequent neutralization (the product is considered active if log $R > 5$).

TABLE 3 Genotypes and phenotypes of mutants for cationic antibacterial compounds[a]

Parent and mutant strain	Selective agent	*norA* mutation	MIC (mg/liter)						MBC (mg/liter)					
			NOR	CIP	EB	AF	CHX	BZC	NOR	CIP	EB	AF	CHX	BZC
ATCC 25923		Wild type	1	1	16	32	2	2	8	1	16	64	2	4
MO060	AF	A94T	16	8	64	256	4	4	16	8	128	256	8	8
MO061	AF	T91A	8	4	32	256	4	4	8	4	64	256	4	4
MO062	AF	T126A	8	4	128	256	4	4	16	4	128	256	32	8
MO072	EB	T126A	8	4	128	256	4	4	8	4	128	256	4	4
MO058	EB	T126A	8	2	128	256	4	4	8	16	128	256	32	16
MO059	EB	T126A	8	4	128	256	4	4	8	4	128	256	4	4
ATCC 6538		Wild type	1	0.5	4	16	4	2	2	0.5	16	32	4	8
MO063	AF	A107G	8	4	32	128	4	4	8	4	32	256	4	4
MO064	AF	T126A	8	2	32	128	4	4	8	4	64	256	4	8
MO065	AF	T126A	8	2	32	128	4	4	8	4	64	256	8	4
MO037	CHX	T89G	16	4	32	64	8	4	16	4	64	128	128	4
MO038	CHX	T89G	16	4	256	256	4	4	16	4	256	256	8	8
MO039	CHX	T90G	8	4	256	256	4	4	8	4	256	256	16	8
MO040	CHX	T90G	8	4	32	64	4	4	16	4	128	64	32	4
MO041	CHX	T90G	8	4	16	256	4	4	8	4	64	256	64	4
RN4220		Wild type	1	0.5	8	16	8	2	2	1	16	32	8	2
MO069	AF	A107G	8	4	64	256	4	4	8	4	128	256	8	8
MO070	AF	A107G	16	8	64	256	4	4	16	8	128	256	8	8
MO071	AF	T126A	8	4	64	128	4	8	16	4	64	256	4	8
MO066	EB	T126A	16	4	128	256	4	4	16	8	128	256	8	8
MO067	EB	A107G	8	4	64	256	4	4	16	4	128	256	8	8
MO068	EB	T126A	16	4	64	256	4	4	16	8	128	256	32	8
MO042	CHX	A130C T126A	8	4	256	256	8	4	16	8	256	256	8	4
MO043	CHX	A130C T126A	16	4	256	256	8	4	16	8	256	256	32	4
MO044	BZC	A130C T126A	16	4	128	256	4	8	16	16	256	256	8	8
MO045	BZC	A130C T126A	16	8	128	256	8	4	16	8	128	256	8	8

[a] NOR, norfloxacin; CIP, ciprofloxacin; EB, ethidium bromide; AF, acriflavine; CHX, chlorhexidine; BZC, benzalkonium chloride.

chlorhexidine (Fig. 1A and E). In the case of the group of strains with MICs to ethidium bromide of up to 32 mg/liter, *qac* determinants were detected only in 12/160 (7.5%) isolates (ethidium bromide-susceptible strains), while clinical strains with low susceptibility to ethidium bromide have a strong association with the presence of *qacA* and *qacB* genes (ethidium bromide MIC of ≥64 mg/liter; $P < 0.001$). The presence of *qacA* increased the mode MIC for ethidium bromide by five dilutions (Fig. 1J). The only *qacG*-carrying strain showed a MIC value of 64 mg/liter, while the MIC values of *qacC*-positive strains did not fall into the group of ethidium bromide-resistant strains but were distributed around an intermediate MIC of 32 mg/liter. The distribution of acriflavine MIC values was similar to that of ethidium bromide (data not shown).

MLST analysis. Among the 91 clinical isolates analyzed, 84 belonged to 13 previously reported STs, while 7 strains showed a new ST due to the presence of at least one novel allele or to the presence of a combination of alleles previously unreported. Almost all strains carrying *qac* genes fell into clonal complex 5, one of the major lineages of nosocomial MRSA. The detailed data on MLST are shown in Table S3 in the supplemental material.

Mutant selection and phenotype analysis. Mutants of *S. aureus* ATCC 6538, ATCC 25923, and RN4220 were selected on a series of cationic antimicrobial substances, including ethidium bromide, acriflavine, benzalkonium chloride, and chlorhexidine. Single-exposure mutants could be selected by acriflavine with frequencies of 2.1×10^{-9} in RN4220, 1.5×10^{-9} in ATCC 25923, and 5.4×10^{-9} in ATCC 6538. A similar frequency was found by selecting ethidium bromide mutants in ATCC 25923 (2.8×10^{-9}), while ethidium bromide mutants had a frequency near the limit of detection in RN4220 (8.1×10^{-10}) and could not be selected in ATCC 6538 ($<1.0 \times 10^{-11}$). For the biocides benzalkonium chloride and chlorhexidine, no single-exposure mutant could be obtained ($<1.0 \times 10^{-11}$). The selection of benzalkonium chloride and chlorhexidine mutants could be achieved only by serial passages on agar plates with increasing concentrations of the biocides (multiple-exposure mutants). Phenotypes of all mutants were comparable irrespective of the selective agent and showed about an 8-fold increase in both MIC and MBC to ethidium bromide and acriflavine. For the two biocides benzalkonium chloride and chlorhexidine, none of the mutants showed any increase in MIC. Only a few of the mutants selected by 5 chlorhexidine showed an increase of the MBC to chlorhexidine only (Table 3). Considering a resistance breakpoint of 1 mg/liter for ciprofloxacin, all mutants, irrespective of the selective agent, were fluoroquinolone resistant (Table 3).

Screening of mutations in the *norA* promoter region. All mutants selected *in vitro* showed mutations in the *norA* promoter region (Table 3 and Fig. 2), many of which were described in the past (9, 11, 33). The T126A mutation, mapping within the −35 site of the predicted *norA* promoter region, was the most frequent mutation found (present in 13/24 mutant strains). All *in vitro*-selected *norA* mutants were resistant to norfloxacin (EUCAST ecological cutoff value for susceptibility [ECOFF] for the wild type of ≤4

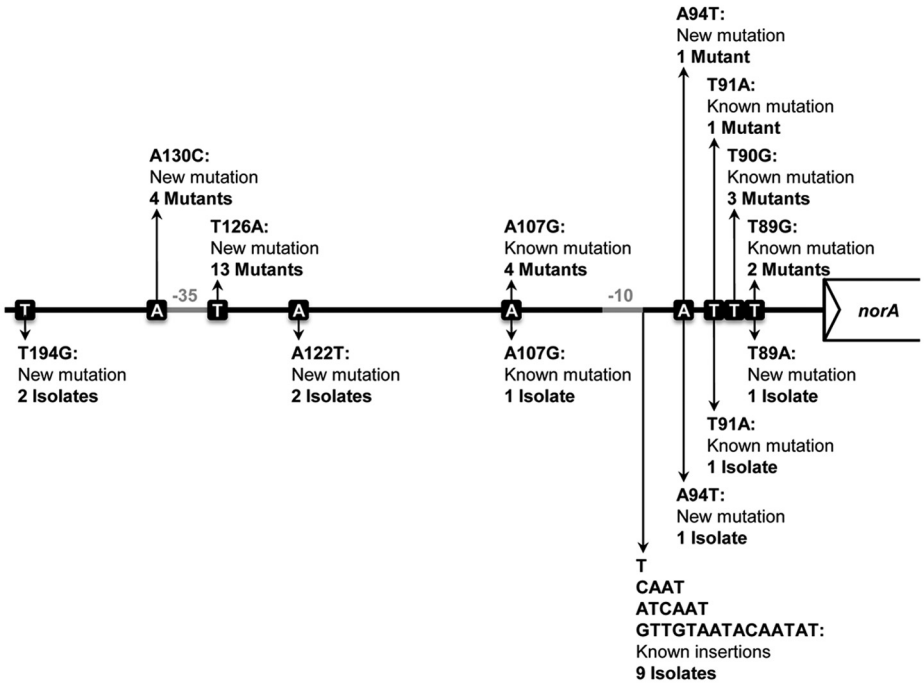

FIG 2 Schematic map of the intergenic region upstream of *norA*. Mutations selected *in vitro* by benzalkonium chloride, chlorhexidine, ethidium bromide, and acriflavine in strains ATCC 6538, ATCC 25923, and RN4220 are shown above the sequence. Mutations deriving from the analysis of the *norA* promoter region of 39 clinical isolates are shown below the sequence. The numbering initiates at the nucleotide in front of the start codon of *norA* and counts from right to left. The putative promoter consensus is shown in gray.

mg/liter) and ciprofloxacin (EUCAST clinical breakpoint $R > 1$ mg/liter). Detection of *norA* promoter mutations was also performed in 39 clinical isolates, including all 11 *qacABCGJ*-negative isolates with EB MICs above 16 mg/liter (Table 4). Twenty-five strains (64%) carried a wild-type allele, and among them, 9 isolates were *qacA* or *qacC* positive. All clinical strains with *norA* mutations were resistant to norfloxacin. Irrespective of their MIC to ethidium bromide, four clinical isolates with mutations in the *norA* promoter region (QBR102278-1027, QBR102278-1191, QBR102278-2634, and QBR102278-2635) were susceptible to ciprofloxacin (Table 4). Notably, the majority of clinical strains which showed changes in *norA* promoter regions had changes distinct from those found in mutant strains.

***Galleria mellonella* infection model.** To evaluate if the low concordance between *norA* mutations selected *in vitro* and those identified in clinical isolates was due to lack of fitness of the laboratory mutants, we performed a virulence test in the greater wax moth larva (*Galleria mellonella*) (32). For this analysis, we compared the virulence of the three reference strains, ATCC 6538, ATCC 25923, and RN4220, to their *norA* promoter mutants (Fig. 3). No decrease in larvicidal capacity was detected for any of the mutants. Growth curves of mutants also did not differ from those of their parental strains (data not shown).

DISCUSSION

Use of biocidal products has increased during recent years, raising concern about possible biocide resistance and even coresistance and cross-resistance to antibiotics. The present work addresses the comparative characterization of efflux mechanisms yielding reduced susceptibility to the cationic biocides benzalkonium chloride and chlorhexidine both *in vitro* and in clinical isolates of *S. aureus*. For the purpose of this study, the standard CLSI MIC and MBC protocols were adopted. They are the only standardized tests available to define bacterial resistance, since the normed tests for biocides are intended to measure activity of the substance or product, not the resistance of target organisms (34, 35).

In *S. aureus*, several transporters involved in biocide efflux have been reported to date, including plasmid-based QacA, QacB, QacC, QacG, and QacJ and the chromosomal NorA (8, 13–15, 17, 18). In our collection of 1,602 *S. aureus* strains of human origin, the frequencies of *qacA* (5.7%), *qacB* (0.3%), *qacC* (3.4%), and *qacG* (0.1%) were consistent with previously reported data (12, 36). In further accordance with published literature, our data show an increase in the mode MIC of benzalkonium chloride in the presence of *qacA*, *qacB*, *qacC*, and *qacG* and also an increase of chlorhexidine mode MIC in the presence of *qacA* and *qacB*. This variation in growth inhibition is in contrast to the complete absence of any effect on the cidal activity of biocides. Others had observed that *qac* genes confer less than a 2-fold decrease in susceptibility, which could have been missed by our assays based on 2-fold dilutions. Still, this technical difference does not explain the absence of any correlation between *qac* genes and biocide MBC observed here (37). Our screening underlines that on a very large set of clinical isolates, none of the *qac* determinants decreased the susceptibility to biocides of staphylococci tested according to CLSI standard bactericidal assays (MBC). The absence of a change in susceptibility is reflected by the absence of variation in biocide activity assayed according to the EN 1276 norm. Since there is absolutely no correlation between increased MBC to both benzalkonium chloride and chlorhexidine and presence of any *qac*

TABLE 4 *norA*-related genotypes in clinical isolates of *S. aureus*

Intergenic region or parent and mutant strain name	Presence of:				MIC[c] (mg/liter)					Sequence of polymorphic site in *norA* promoter region[a]	Comment[b]
	qacA	*qacB*	*qacC*	*qacG*	NOR	CIP	EB	BZC	CHX		
Intergenic region											
1										1111111111----------9999998864332222876 1	
2										9543322100 9754107415639820	
3										4052062270	
Strain											
MW2	−	−	−	−	ND	0.5	8	1	2	TAT-ATAGAT----------AAAATTTGCGCTCATGGTGT	wt allele
Mu50	+	−	−	−	ND	64	256	4	4	...-.....----------.....	wt allele
COL	+	−	−	−	ND	64	128	4	4	...-.....----------.....	wt allele
RN4220	−	−	−	−	1	0.5	8	2	8	...-.....----------.....	wt allele
ATCC 6538	−	−	−	−	1	0.5	4	2	4	...-.....----------.....	wt allele
1016	−	−	−	−	ND	0.25	8	2	2	...-.....----------.....	wt allele
1025	−	−	−	−	ND	1	8	2	2	...-.....----------.....	wt allele
1130	−	−	−	−	ND	0.25	32	2	2	...-.....----------.....	wt allele
1170	−	−	−	−	64	64	4	8	2	...-.....----------.....	wt allele
1173	+	−	−	−	64	64	256	4	2	...-.....----------.....	wt allele
1177	−	−	−	−	64	64	8	1	2	...-.....----------.....	wt allele
1192	+	−	−	−	ND	64	256	8	4	...-.....----------.....	wt allele
1205	+	−	−	−	64	0.5	256	8	2	...-.....----------.....	wt allele
1209	−	−	+	−	8	0.25	32	8	2	...-.....----------.....	wt allele
1219	+	−	−	−	ND	8	256	8	4	...-.....----------.....	wt allele
1236	−	−	−	−	2	0.25	16	8	2	...-.....----------.....	wt allele
1284	−	−	−	−	ND	0.25	8	2	1	...-.....----------.....	wt allele
1299	−	−	+	−	2	0.25	16	4	1	...-.....----------.....	wt allele
1390	−	−	+	−	64	2	32	8	1	...-.....----------.....	wt allele
1407	−	−	−	−	ND	0.25	2	1	1	...-.....----------.....	wt allele
1503	+	−	−	−	ND	8	128	4	2	...-.....----------.....	wt allele
1505	+	−	−	−	ND	8	128	4	4	...-.....----------.....	wt allele
1508	+	−	−	−	ND	8	128	4	2	...-.....----------.....	wt allele
1522	+	−	−	−	ND	8	128	4	2	...-.....----------.....	wt allele
1619	−	−	−	−	2	0.25	4	2	2	...-.....----------.....	wt allele
1730	−	−	−	−	32	8	4	8	4	...-.....----------.....	wt allele
1828	−	+	−	−	2	0.25	256	8	2	...-.....----------.....	wt allele
1889	−	−	+	−	64	64	64	8	1	...-.....----------.....	wt allele
2092	−	−	+	−	ND	0.25	32	8	2	...-.....----------.....	wt allele
2106	+	−	−	−	64	8	128	8	4	...-.....----------.....	wt allele
2363	−	−	−	−	ND	64	32	4	2	...-.....----------.....	wt allele
2391	−	−	−	−		64	32	4	8	...-.....----------.....	wt allele
2507	−	+	−	−	1	0.25	512	8	2	...-.....----------.....	wt allele
2577	−	−	−	−	2	0.25	4	64	8	...-.....----------.....	wt allele
2628	+	−	−	−	64	0.25	128	8	8	...-.....----------.....	wt allele
2671	+	−	−	−	2	0.25	128	8	4	...-.....----------.....	wt allele
1191	−	−	−	+	128	0.25	64	4	2	...-....**G**.----------.....	A107G, known mutation (9)

ATCC 25923	−	−	−	1	1	16	2	2	...A.....----------.....T.A.A.....		wt allele
1285	−	−	−	64	0.25	8	2	2	...A.....----------.....T.A.A.....		wt allele
1027	−	−	−	128	0.25	16	16	4	**G..**-.....----------.....		T194G, new mutation
1158	+	−	−	128	8	64	8	4	**G**..-.....----------.....		T194G, new mutation
1614	−	−	+	64	2	64	8	2	.GC-.....----------.....		A150G, T145G, new mutations
1387	−	−	−	64	16	4	4	2	...-.....----------**ATCAAT**.....	---.	Known duplication (11), new deletion
1607	−	−	−	64	32	16	4	2	...-.....**GTTGTAATACAATAT**.....		New duplication
1881	−	−	−	64	64	32	8	4	...-.....----------**CAAT**.....		Known duplication (11)
1891	−	−	−	64	64	32	8	4	...-.....----------**CAAT**.....		Known duplication (11)
1939	−	−	−	64	64	32	8	4	...-.....----------**CAAT**.....		Known duplication (11)
1951	−	−	−	64	64	32	4	4	...-.....----------**CAAT**.....		Known duplication (11)
1878	−	−	−	64	64	8	4	2	...-...A..----------**CAAT**.....A.GAGAT...A		Known duplication (11)
1894	+	−	−	64	64	16	4	2	...-...A..----------**CAAT**.....A.GAGAT...A		Known duplication (11)
2345	−	−	+	16	0.25	32	2	2	...-...A..----------.....A.GAGAT...A		wt allele
2635	−	−	−	8	0.5	128	8	4	...A..**T**A..----------**T**.....A..AGAT...A		New duplication; A122T, new mutation
2605	−	−	−	16	64	64	4	2	...A..TA..----------.....A..AGAT...A		A122T, new mutation
2634	−	−	−	8	0.5	32	8	4	...A..**T**A..----------.TC**TAA**G-.A..AGAT...A		T91A, known mutation (22); A122T, A94T, T89A, new mutations; new deletion
1277	−	−	−	64	0.5	8	2	2	...A...A..----------.....A..AGAT...A		wt allele

[a] Polymorphic sites are indicated with respect to the *norA* promoter region sequence of *S. aureus* MW2. The first three rows provide the numbering of the intergenic regions. The intergenic regions start from the nucleotide upstream of the *norA* start codon (NC_003923 position 739144) and are numbered backward (from right to left). Nucleotides that were the same as those of MW2 in all sequences are not shown. Dots indicate perfect homology with the reference sequence. Insertions and deletions are marked with dashes. Mutations probably involved in *norA* overexpression are in boldface.

[b] wt, wild type.

[c] ND, not determined.

determinant but the correlation between increased MBCs is statistically significant, reduced susceptibility to both compounds should have the same molecular mechanism. So far, our data showed that this is not linked to the presence of *qac* genes, and preliminary data on four strains also exclude upregulation of other known efflux systems. This leaves the issue of the molecular mechanism of increased biocide MBC in staphylococci open to speculation.

This analysis of 1,602 isolates for MIC and MBC, the largest set of staphylococcal strains ever analyzed, was unable to uncover a clear indication of an ECOFF (38) or breakpoint for resistance to both benzalkonium chloride and chlorhexidine. In contrast, ethidium bromide screening showed a clear cutoff between a susceptible *S. aureus* population and nonsusceptible strains and a perfect match to the presence of *qacA* and *qacB* genes (6). This is in keeping with efflux pump overexpression being effective for compounds acting inside the cell and being less effective on biocides targeting the membrane(s) and acting from outside the cell (20). Our recent analysis of triclosan susceptibility using the same set of isolates showed a clear ECOFF for triclosan (22). No correlation of reduced susceptibility to triclosan and benzalkonium chloride and chlorhexidine (coresistance) was detected in our data set, even if recent reports have identified plasmids carrying both *qacA* and the newly described sh-*fabI* allele (22, 39, 40).

European legislation on biocide registration is changing, and a test for risk assessment for biocide resistance has been proposed. In this context, we have investigated the correlation between the molecular nature of reduced susceptibility in clinical isolates and mutations selected *in vitro*. We selected *S. aureus* mutants with reduced susceptibility to a series of compounds, all previously linked to efflux by the NorA efflux pump. In our assays, mutants with reduced susceptibility could be selected by all compounds, although the biocides required multiple passages. All mutants showed mutations in the *norA* promoter region and the same efflux phenotype. As observed in clinical isolates in our *in vitro* mutants, ethidium bromide and acriflavine susceptibility profiles changed significantly, while those for benzalkonium chloride and chlorhexidine were quite similar to those of wild-type strains (6). The fact that mutations conferred only a limited increase in resistance to biocides is the most probable reason for the failure to select, with a standard one-step protocol, mutants with these biocides. Of the 14 clinical isolates in which we had found polymorphisms in the promoter region of *norA*, eight evidenced short direct repeats in the promoter region (12). In contrast, in our *in vitro* mutant strains, these duplications were not present, even though *in vitro* selection has been reported to occur (11). Among the point mutations selected *in vitro*, only 14% (4/28) matched those in clinical isolates, which in turn represented only 21% (3/14) of the total number of mutated clinical isolates. These data indicate quite clearly that an *in vitro* test, such as the one carried out here, would have a very low predictive value for clinically relevant reduced biocide susceptibility. Approaches involving shorter contact times and, possibly, neutralization of the biocides may be explored for further investigation into *in vitro* tests for prediction of biocide resistance. So far, the most suggestive explanation for the difference of mutations *in vitro* and in clinical isolates is the absence of selective pressure for fitness *in vitro*. In the absence of a validated fitness model, the killing of wax moth larvae had been proposed to serve as a fitness assay for biocide mutants in staphylococci (26, 41). Even having screened 13 independent mu-

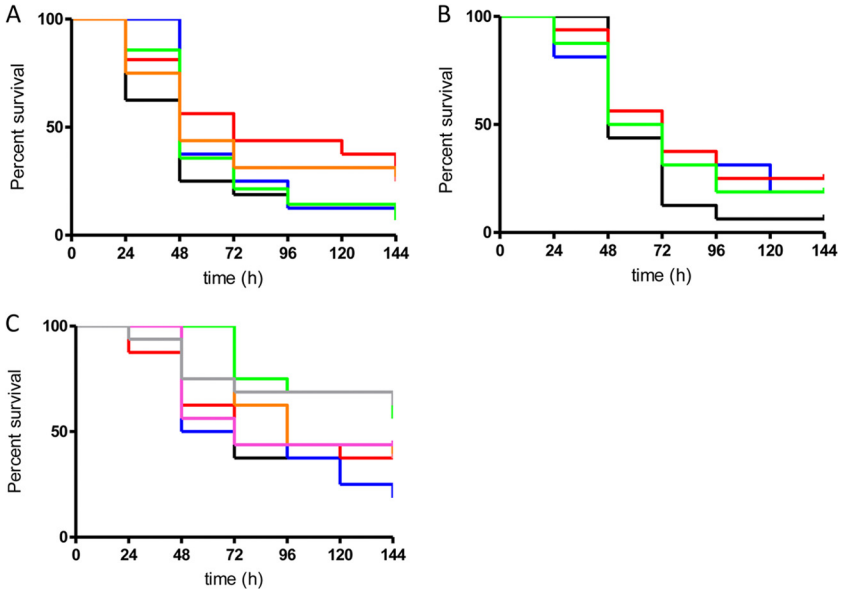

FIG 3 Fitness assay in *Galleria mellonella* larvae. *S. aureus* strains with mutations in the *norA* promoter region were evaluated for their fitness in *G. mellonella* killing experiments (26). In each experiment shown, 16 larvae where infected with 10^5 CFU/larva of mutants selected from strain ATCC 6538 (A), ATCC 25923 (B), and RN4220 (C). The single strains shown in panel A are ATCC 6538 (black), MO037 (blue), MO039 (red), MO063 (green), and MO064 (orange); in panel B they are ATCC 25923 (black), MO060 (blue), MO061 (red), and MO062 (green); and in panel C they are RN4220 (black), MO043 (blue), MO045 (red), MO066 (green), MO067 (orange), MO069 (pink), and MO071 (gray). A statistically significant reduction of virulence was evidenced using the log-rank test for any of the mutants.

tants in three different *S. aureus* strains, we could not identify any phenotype of reduced *in vivo* fitness.

In summary, our data show that 9.5% of clinical isolates of *S. aureus* carry known genes associated with ethidium bromide efflux and reduced susceptibility to biocides. Fine characterization of the substrate specificity of these pumps associates (i) mutations of the promoter region of *norA* with a 2-fold increase in MIC to both benzalkonium chloride and chlorhexidine, (ii) the presence of the plasmid-encoded MFS pumps QacA and QacB with a 4-fold increase in MIC of benzalkonium chloride and 2-fold increase in MIC of chlorhexidine, and (iii) the plasmid-encoded SMR efflux pumps QacC and QacG with a 2-fold increase in MIC to benzalkonium chloride and no increase in MIC to chlorhexidine. Regarding cross-resistance to antibiotics *in vitro*, mutation of the *norA* promoter conferred cross-resistance to norfloxacin and ciprofloxacin, but not all clinical isolates showing *norA* promoter mutations were resistant to ciprofloxacin, and none of the plasmid-encoded efflux pumps conferred resistance to antibiotics. Importantly, data from clinical isolates has shown that none of these determinants has any effect on the bactericidal activity of biocides when using either CLSI assays or the EN 1276 norm. This does not indicate that these transporters do not contribute to efflux of their known substrates, but that the specific effect of these MDR pumps is evidenced in *S. aureus* exclusively utilizing the CLSI MIC growth inhibition assay. The importance given to the cidal effect of biocides in many contexts, inducing the discussion of their clinical relevance in selecting for antibiotic resistance, the recommended in-use concentrations, which are far above the natural resistance of bacteria, and the planning of resistance surveys, may have to be critically revised. At least in the specific case of benzalkonium chloride and chlorhexidine and when using the *S. aureus* model, we suggest focusing efforts only on MIC assays when trying to correlate biocides and antibiotics susceptibility profiles to the relative resistance genes during hazard evaluation and risk assessments (2). Given our data, such a simplification is completely justified and facilitates high-throughput screening. The resulting increase in numbers and reduction in cost in the presence of an unaltered capacity of resistance prediction will allow us to significantly speed up work on the risk evaluation of the use of these compounds. On the other hand, our data suggest, with regard to a possible introduction of tests for risk assessment for benzalkonium chloride and chlorhexidine resistance in *S. aureus*, that current *in vitro* tests for resistance development have a poor predictive value and low clinical relevance.

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