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Genetic changes associated with tigecycline resistance in *Staphylococcus aureus* in vitro-selected mutants belonging to different lineages

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ABSTRACT

Tigecycline (TGC) resistance remains rare in *Staphylococcus aureus* worldwide. In this study, 12 TGC-resistant *S. aureus* mutants (TRSAm) were obtained displaying an increase in efflux activity. The isolates belonged to seven different genetic lineages, with a predominance of clonal complex 5 (CC5). Diverse genetic changes in *mepA* and *mepR* genes were found producing alterations in the amino acid sequences of the corresponding proteins (MepA and MepR, respectively). The most frequent amino acid change in MepA was Glu287Gly. All of the TRSAm exhibited different single nucleotide polymorphisms (SNPs) or insertions/deletions (InDels) in *mepR* causing premature stop codons or amino acid changes in MepR. Expression of *mepA* was significantly increased in TRSAm with different mutations in *mepA* and *mepR*. Of the 12 TRSAm, 6 also harboured mutations in *rpsJ* that resulted in amino acid changes in the S10 ribosomal protein, with Lys57 being the most frequently mutated site. Our findings demonstrate that these acquired mechanisms of TGC resistance are not restricted to a single type of genotypic background and that different lineages might have the same plasticity to develop TGC resistance. The impact of TGC selective pressure assessed by whole-genome sequencing in four selected strain pairs revealed mutations in other singular genes and IS*256* mobilisation.

1. Introduction

Staphylococcus aureus is one of the major pathogens causing serious infections both in hospital and community settings. This micro-organism is characterised by an extraordinary genomic plasticity and the ability to adapt to different environments and acquire new antibiotic resistance determinants.

Over the years, *S. aureus* has acquired several genetic determinants of antimicrobial resistance, thus treatment options are becoming more limited. Currently, vancomycin is the therapeutic choice in severe infections caused by methicillin-resistant *S. aureus* (MRSA), but since 1997 vancomycin-intermediate *S. aureus* (VISA) strains have emerged causing great concern worldwide [1].

Tigecycline (TGC) is a bacteriostatic antibiotic derived from minocycline belonging to the glycylcycline class of antibiotics with broad-spectrum in vitro activity. TGC has been approved by the US Food and Drug Administration (FDA) for the treatment of complicated intra-abdominal infections, complicated skin and skin-structure infections and community-acquired bacterial pneumonia. It represents a useful therapeutic option for the treatment of complicated infections caused by multidrug-resistant organisms with limited treatment options [2].

TGC surveillance studies conducted since its approval continue to show good activity against *S. aureus*, with a high susceptibility percentage (99.9%) reported in several countries around the world [3]. In Argentina, the first case of an *S. aureus* clinical isolate non-susceptible to TGC was described in 2017, recovered from a cystic fibrosis patient in which TGC susceptibility decreased after an extended period of complex antimicrobial therapy including minocycline [4].

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TGC evades tetracycline resistance mechanisms such as the ribosomal protection provided by TetM because it binds to the bacterial ribosome with a different orientation than classical tetracyclines [5].

Acquired resistance to TGC in various bacterial genera is usually associated with overexpression of efflux pumps. Several efflux pumps are encoded on the *S. aureus* chromosome with different abilities to extrude antimicrobial compounds. The NorB efflux pump, a member of the major facilitator superfamily (MFS), has the ability to expel tetracyclines, fluoroquinolones and dyes [6]. The MepA efflux pump, belonging to the multidrug and toxic compound extrusion (MATE) family of multidrug efflux pumps, was previously associated with reduced susceptibility to TGC in *S. aureus* mutants [7].

The *mepA* gene forms part of the *mepRAB* operon, which also comprises the *mepR* gene encoding a transcriptional regulator (MepR) and the *mepB* gene whose product possesses endonuclease activity [7,8].

Several mutations that affect the functional capacity of MepR as well as mutations in MepA have been indicated as responsible for the acquisition of TGC resistance in *S. aureus* [7,9]. In addition, mutations in the *rpsJ* gene encoding the S10 ribosomal protein have also been reported in TGC-resistant *S. aureus* isolates and other bacterial species [4,10,11].

The aim of this work was to perform a comparative analysis of clinical *S. aureus* isolates belonging to different genetic lineages and their derived resistant mutants obtained under TGC selective pressure in order to unravel the phenotypic and genotypic changes associated with TGC resistance.

2. Methods

2.1. Clinical isolates

A total of 20 Argentinean *S. aureus* clinical isolates, including 10 methicillin-susceptible *S. aureus* (MSSA) and 10 MRSA, from our laboratory collection but without an epidemiological link were included in this study.

2.2. In vitro selection of tigecycline-resistant mutants

In vitro mutant selection was performed by serial passage in Mueller–Hinton (MH) broth (Britania, Argentina) with increasing concentrations of TGC (Pfizer, USA) following a previously described procedure [12]. Mutant stability was evaluated by determining the minimum inhibitory concentration (MIC) of TGC after 10 consecutive passages in antibiotic-free tryptic soy agar medium (Britania).

The clonal relationship between the parental and mutants strains was confirmed by pulsed-field gel electrophoresis (PFGE) using *Sma*I endonuclease [12].

2.3. Antibiotic susceptibility testing

The MIC of TGC was determined by Etest and was interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [13]. MICs to other antibiotics were determined by the agar dilution method following Clinical and Laboratory Standards Institute (CLSI) recommendations [14], including oxacillin, cefoxitin, trimethoprim/sulfamethoxazole, rifampicin, erythromycin (Sigma-Aldrich, USA), vancomycin, gentamicin, ciprofloxacin (Fada Pharma, Argentina) and doxycycline (Chemo S.A., Lugano, Switzerland). *Staphylococcus aureus* ATCC 29213, ATCC 43300 and ATCC 700699 (Mu50) were used as control strains.

2.4. Genotypic characterisation of Staphylococcus aureus strains

The staphylococcal cassette chromosome *mec* (SCC*mec*) type was determined in MRSA isolates by characterisation of the *ccr* complex (cassette chromosome recombinase) and the *mec* complex using a simplified version of a previously describe scheme [15,16]. Moreover, isolates were genotyped by *spa* typing [17] and multilocus sequence typing (MLST) using the *S. aureus* MLST database (https://pubmlst.org/organisms/staphylococcus-aureus). The *agr* type was characterised by multiplex PCR [18].

2.5. Fitness evaluation

To determine whether the acquisition of TGC resistance was associated with a fitness cost, growth curves, mutation frequency and autolysis assays were performed for each TGC-resistant *S. aureus* mutant (TRSAm) and its counterpart parental strain. These experiments were performed in triplicate.

2.5.1. Growth curves

A fresh culture of each strain (dilution 1/1000) was grown in MH broth and incubated at 37°C and 180 rpm until reaching an optical density at 620 nm (OD_{620}) of 0.1. From that moment on, the OD_{620} and the CFU count were determined every 30 min for 4 h. A growth curve was constructed plotting the log CFU/mL variation over time. Growth rates were statistically compared through slope analysis by linear regression using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA). The significance level was set at $P < 0.05$.

2.5.2. Mutation frequency assay

The mutation frequency assay was performed from a 1/1000 dilution of fresh culture in MH broth and incubated at 37°C at 180 rpm until reaching exponential growth phase (OD_{620} = 0.5–0.7). Then, an inoculum was seeded on MH agar (Britania) plates containing rifampicin (100 μg/mL) and incubated at 37°C overnight. The spontaneous mutation frequency was calculated as the ratio between CFU/mL that grew on antibiotic- and non-antibiotic-containing plates (total number of viable cells). Student's test was applied for statistical analysis, with a significance level of $P < 0.05$.

2.5.3. Autolysis profile

Strains were grown in the MH broth until they reached an OD_{620} of 0.5–0.7. Cells were then chilled in an ice-bath for 10 min, harvested by centrifugation at 5000 rpm for 10 min, and washed with ice-cold distilled water. Cells were then suspended to OD_{620} = 1.0 in lysis buffer [50 mM glycine buffer (pH 8.00), 0.01% Triton X-100]. Autolysis was evaluated by measuring the OD decline every 30 min for 4 h.

2.6. Efflux activity evaluation

Efflux activity was studied phenotypically by comparing the MICs to TGC and ethidium bromide (EtBr) in the presence and absence of 20 μg/mL reserpine (RS) as previously described. An EtBr MIC of $\geq$32 μg/mL coupled with a reduction of at least four two-fold dilutions (TFD) in the MICs of EtBr and TGC in the presence of RS was considered to be indicative of enhanced efflux activity [12].

2.7. Amplification and sequencing of genes encoding the S10 ribosomal protein and efflux pumps

The *rpsJ, norB, mepA* and *mepR* (coding for a *mepA* transcriptional regulator) genes of all TRSAm and parental strains were amplified by PCR and sequenced. The nucleotide and predicted

Table 1
Primers used for PCR amplification of *mepA, mepR, norB, rpsJ* and IS*256*

Primer	Sequence (5′→3′)	Product size (bp)	Reference
mepA-F	CACTCGTATCGCAGTTATCTG	1700	This study
mepA-R	CTTTAACTTCTGATTCTTCACTA		
mepR-F2	CAATAAATGGAATTCACTTATTCG	539	[7]
mepR-R2	CTTTCATTGTTCAATACTCCTTG		
norB-F	GGAGGCTTATCTAATTCATGG	1400	This study
norB-R	CCTAACATTAAACGTCAATACGC		
rpsJ F	AAGCGATGAAGCGAAAGG	500	This study
rpsJ R	CCGAATACTTGTGTCATCCC		
IS*256*-1	CCGACAAAGTCAACGAAA	922	[26]
IS*256*-2	GGCTGATGTTTGATTGGG		

amino acid sequences were analysed using Vector NT 11.0 software and NCBI BLAST (https://www.ncbi.nlm.nih.gov/blast/). The primers used in this study are described in Table 1.

2.8. mepA *and* norB *expression level analysis by reverse transcription quantitative real-time PCR (RT-qPCR)*

Two TRSAm and their corresponding parental strains (94159m/94159p and 2028m/2028p) were selected to analyse *mepA* and *norB* expression by RT-qPCR. Strains were selected considering not only the increase in TGC MICs shown by mutant strains but also the efflux activity in the presence of RS, the different genetic lineages and the differences in mutations in *mepR*.

RNA was isolated by triplicate, in three independent experiments, from bacteria grown in brain–heart infusion broth (Oxoid, USA) until they reached an OD_{620} of 0.5–0.7. Cells were collected by centrifugation and treated with 15 mg/mL lysozyme (Sigma-Aldrich) in 10 mM Tris–HCl (pH 8.00) and 0.1 mM ethylene diamine tetra-acetic acid (EDTA) for 1 h at 37°C. RNA was extracted using TRIzol™ reagent (Invitrogen) with a PureLink® RNA Mini Kit (Ambion) according to the manufacturer's recommendations and was quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific) and treated with 3 U/mL DNAse for 1 h at 37°C (RQ1 RNase-Free DNase; Promega). Reverse transcription was performed using 500 ng of RNA, 200 U of M-MLV™ Reverse Transcriptase (Invitrogen) and 50 μM random primers (Invitrogen) according to the manufacturer's recommendations. The qPCR reaction was carried out using a 1/100 dilution of cDNA, SYBR® SelectMaster Mix (Applied Biosystems) and the primers described by Couto et al. [19] in a 7500 Real-Time PCR System (Applied Biosystems).

The combination of *gyrB* and *pta* was used as reference genes. Cq values were converted into normalised relative quantity values using normalisation to the geometric average of the reference genes and the specific PCR efficiency for each gene [20].

Student's test was applied for statistical analysis, and *P*-values of <0.05 were considered statistically significant. The complete RT-qPCR protocol is described in Supplementary material MIQE.

2.9. Whole-genome sequencing (WGS) and bioinformatics analysis

Differences in sequence types (STs), diverse mutations in *mepA* and *mepR*, and changes in the MIC of antibiotics other than TGC were the criteria considered to select the four pairs of isolates for this purpose. WGS of isolates 94159p, 94159m, 497p, 497m, 74073p, 74073m, 2028p and 2028m was carried out using an Illumina MiSeq platform.

Total bacterial DNA was extracted from overnight cultures using a MasterPure™ Complete DNA and RNA Purification Kit (Epicenter, Madison, WI, USA) according to the manufacturer's instructions, with the addition of 0.03 μg/μL lysostaphin in the lysis step, and incubating for at least 30 min at 37°C.

Sequencing libraries were constructed using an Illumina TruSeq Nano DNA HT Sample Preparation Kit. Sequenced reads were de novo assembled using SPAdes Assembler v.3.9.1 (cab.spbu.ru/software/spades/). Contigs were annotated with Prokka v.1.12-beta [21]. The summary statistics for the assembled draft genomes are shown in Supplementary Table S1. Paired-end reads were mapped to a closely related reference genome, namely ST5 *S. aureus* N315 (GenBank accession no. **BA000018.3**) for 94159p/94159m, 497p/497m and 74073p/74073m and ST239 *S. aureus* TW20 (GenBank accession no. **NC_017331**) for 2028p/2028m.

Single nucleotide polymorphisms (SNPs) were detected and annotated using Snippy v.3.2 software (https://github.com/tseemann/snippy).

Manual inspection of SNPs and insertions/deletions (InDels) was performed using Artemis and/or Integrative Genomics Viewer (IGV) [22,23], and comparative analysis between parent–mutant pairs was performed with ACT/GView [24].

ISseeker software [25] was used to explore the genome to detect differences in insertion sequence (IS) content between parental and mutant strains and also to annotate the flanking edges of IS elements in draft genomes. Relevant InDels mediated by IS*256* were confirmed by PCR (primers described in Table 1).

2.10. Data access

Short reads for all sequenced isolates have been submitted to the NCBI under project accession **PRJNA577848** (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA577848).

3. Results

All *S. aureus* clinical isolates were susceptible to TGC (MIC range, 0.064–0.125 μg/mL), vancomycin (MIC range, 0.5–1 μg/mL) and doxycycline (MIC range, 0.06–4 μg/mL) (Table 2; Supplementary Table S2). Using the same assay for the in vitro selection of TGC-resistant mutants, 12 TRSAm were obtained (5 MRSA and 7 MSSA) that showed a 16- to 128-fold increase in their MIC (MIC range, 1–16 μg/mL) compared with their parental strain. According to the *Sma*I macrorestriction profile, the 12 TRSAm selected were isogenic to their parental strains (Supplementary Fig. S1).

Susceptibility to other classes of antibiotics was tested in order to detect variations in the resistance profile as a result of TGC pressure (Table 2). Of the 12 TRSAm strains, 2 (497m and 94159m) also showed modifications in their susceptibility to other antibiotics such as oxacillin and cefoxitin (Table 2). Moreover, the 94159m strain exhibited an increase in vancomycin resistance, being categorised as VISA [12].

Molecular characterisation revealed that all MRSA isolates harboured the *mecA* gene (encoding the alternative penicillin-binding protein PBP2a) and distinct SCC*mec* types. Seven different STs were detected in this group of isolates corresponding to six clonal complexes (CC): four of the five MRSA isolates belonged to CC5 (ST5 and ST100) and one to CC8 (ST239). The clonal complexes detected in MSSA isolates were CC1 (ST188; 3 isolates), CC45 (ST45; 2 isolates), CC6 (ST6; 1 isolate) and CC30 (ST30; 1 isolate). The most frequent *spa* types were t002 and t189 and the most frequent *agr* group was *agr* I (7/12) followed by *agr* II (4/12), while only 1 isolate belonged to *agr* III (Table 3).

Overall, bacterial fitness assessment did not show important differences between mutant and parental strains. TRSAm showed a trend towards a slower growth rate compared with parental strains, but the differences were statistically significant in only two cases (497p/497m, $P = 0.0257$; and 4261p/4261m, $P = 0.0011$) (Supplementary Fig. S2). In the same way, only two TRSAm showed

Table 2
Susceptibility profile of parental (p) and mutant (m) strains

Strain	MIC (μg/mL)											
	OXA	FOX	VAN	GEN	CIP	ERY	SXT	RIF	TGC	TGC + RS	EtBr	EtBr + RS
497p	**8**	16	1	0.5	0.5	0.25	0.5/9.5	0.015	**0.125**	0.016 [3]	16	0.5 [5]
497m	**1**	32	1	0.5	1	0.25	0.5/9.5	0.015	**4**	0.064 [6]	64	2 [5]
2028p	≥32	≥128	1	≥64	≥16	≥64	≥16/304	≥16	**0.125**	0.064 [1]	16	2 [3]
2028m	≥32	≥128	1	≥64	≥16	≥64	≥16/304	≥16	**16**	0.25 [6]	64	2 [5]
94159p	**8**	**16**	**1**	≥64	0.5	0.25	0.5/9.5	4	**0.125**	0.064 [1]	16	1 [4]
94159m	**64**	**64**	**4**	≥64	0.5	0.25	0.5/9.5	4	**16**	1 [4]	128	8 [4]
74016p	4	32	0.5	≥32	16	0.25	0.5/9.5	4	**0.125**	0.064 [1]	32	0.25 [7]
74016m	8	32	1	32	16	0.5	0.5/9.5	4	**4**	0.125 [5]	64	2 [5]
4261p	8	16	1	32	≥16	0.25	0.5/9.5	4	**0.064**	0.032 [1]	16	0.25 [6]
4261m	16	32	1	32	≥16	0.5	0.25/4.75	8	**8**	0.125 [6]	64	4 [4]
54081p	0.125	≤0.5	1	1	0.25	0.25	0.5/9.5	0.007	**0.064**	0.032 [1]	16	1 [4]
54081m	0.125	≤0.5	1	0.5	0.5	0.25	0.5/9.5	0.015	**8**	1 [3]	64	8 [3]
74073p	0.5	4	1	0.5	0.5	0.5	0.25/4.75	≥8	**0.064**	0.064 [0]	32	1 [5]
74073m	0.5	4	1	0.5	1	0.25	0.25/4.75	≥8	**8**	0.25 [5]	32	2 [4]
14069p	0.5	4	1	0.25	1	0.25	0.125/2.375	0.007	**0.064**	0.064 [0]	16	0.5 [5]
14069m	0.25	4	1	0.5	0.5	0.5	0.25/4.75	0.015	**1**	0.064 [4]	64	8 [3]
34076p	0.125	2	1	0.25	1	0.25	0.25/4.75	0.015	**0.125**	0.064 [1]	16	1 [4]
34076m	0.125	4	1	0.5	2	0.5	0.125/2.375	0.015	**16**	0.25 [6]	64	2 [5]
34204p	0.25	4	1	0.25	0.5	≥32	0.25/4.75	0.007	**0.064**	0.064 [0]	16	0.5 [5]
34204m	0.5	4	1	0.25	0.5	≥32	0.125/2.375	0.015	**8**	0.5 [4]	32	1 [5]
44213p	0.25	4	1	0.5	0.5	0.25	0.25/4.75	0.007	**0.125**	0.064 [1]	16	0.5 [5]
44213m	0.5	4	1	0.25	0.5	0.5	0.25/4.75	0.007	**4**	0.125 [5]	32	0.25 [7]
34023p	0.5	4	0.5	0.25	0.5	0.5	0.25/4.75	0.007	**0.064**	0.064 [0]	8	0.5 [4]
34023m	0.5	4	1	0.5	1	0.5	0.25/4.75	0.015	**1**	0.125 [3]	32	4 [3]

MIC, minimum inhibitory concentration; OXA, oxacillin; FOX, cefoxitin; VAN, vancomycin; GEN, gentamicin; CIP, ciprofloxacin; ERY, erythromycin; SXT, trimethoprim/sulfamethoxazole; RIF, rifampicin; TGC, tigecycline; RS, reserpine; EtBr, ethidium bromide.
NOTE: MIC differences between the parental and mutant strains are indicated in bold.
The number of dilutions that the MIC decreased in the presence of RS is shown in brackets.

Table 3
Molecular characterisation of *Staphylococcus aureus* parental and mutant strains

Strain (parental and mutant)	SCC*mec*	MLST		*spa* type	*agr* group
		ST	CC		
497	IV	5	5	t311	II
2028	III	239	8	t654	I
94159	IV	100	5	t002	II
74016	NT	100	5	t002	II
4261	IV	100	5	t002	II
54081	NA	45	45	t330	I
74073	NA	6	6	t701	I
14069	NA	188	1	t189	I
34076	NA	45	45	t230	I
34204	NA	188	1	t189	I
44213	NA	30	30	t021	III
34023	NA	188	1	t189	I

SCC*mec*, staphylococcal cassette chromosome *mec*; MLST, multilocus sequence typing; ST, sequence type; CC, clonal complex; NT, non-typeable; NA, not applicable.

statistically significant differences in autolysis activity, being lower in mutant strains (74073p/74073m, $P = 0.011$; and 44213p/42213m, $P = 0.002$). No change in mutation frequency was observed (Supplementary Table S3).

Efflux activity was evaluated phenotypically as a possible mechanism of resistance to TGC in TRSAm. A reduction of at least four TFD in the TGC MIC in the presence of RS was observed in 10 of 12 mutants. Moreover, the same effect of RS on EtBr MICs was observed in 9 of 12 mutants. A reduction of three TFD in the MIC values was detected for the rest of the mutants (Table 2). These results suggest that the TGC resistance mechanism is related to an increase in efflux activity.

Diverse genetic changes in *mepA* and *mepR* genes were found in TRSAm, with predicted alterations in the amino acid sequences of the corresponding proteins (MepA and MepR, respectively). No changes were found in *norB*. Table 4 summarises the alterations found in the nucleotide sequences of *mepA* and *mepR* and their translated amino acid sequences.

The most frequent amino acid change detected in MepA (Glu287Gly) was found in 6/12 TRSAm of five different genetic lineages (CC1, CC5, CC6, CC8 and CC45). Moreover, 4/12 TRSAm [CC5 (2), CC8 and CC45] harboured the Thr29Ile mutation. Also, 3/12 TRSAm strains [CC5 (2) and CC45] harboured the Leu288Phe mutation. Other non-repeated amino acid changes in MepA are displayed in Table 4.

All of the TRSAm exhibited different SNPs/InDels in *mepR* causing premature stop codons or amino acid changes without any conserved mutation (Table 4). Three of the mutants presented InDels in *mepR*.

Levels of expression of *mepA* and *norB* genes were determined by RT-qPCR in two pairs of parental/mutant strains (94159m/94159p and 2028m/2028p). In both cases, the *mepA* transcript level was significantly increased in TRSAm strains (Fig. 1; $P < 0.001$). Moreover, transcription of the *norB* gene was lower in both TRSAm, being significantly different only for the 2028m/2028p pair (Fig. 1; $P = 0.0007$).

Of the 12 mutants, 6 showed nucleotide substitutions in the *rpsJ* gene that rendered amino acid changes in the S10 ribosomal protein between positions 53–60, with Lys57 being the most frequently mutated site (Table 4).

WGS of four selected parental/mutant pairs (94159p/94159m, 497p/497m, 74073p/74073m and 2028p/2028m) were analysed to detect additional genetic changes associated with selection for TGC-resistant variants.

By WGS analysis, the genetic changes described above for *mepA, mepR* and *rpsJ* genes were confirmed and a mutation in the *rpsN* gene (Arg23His) encoding another 30S ribosomal protein (S14) was detected in strain 74073m, which might therefore be associated with TGC resistance (Supplementary Table S4). Other mutations were found in singular genes exhibiting a different pattern in each strain. However, mutation in *fakA* (or *vfrB*) was detected in 3/4 TRSAm. This gene encodes a protein of the dihydroxyacetone kinase family that is related to the regulation of virulence factors [27,28] (Supplementary Table S4).

Table 4
Mutations found in tigecycline-resistant *Staphylococcus aureus* mutants associated with tigecycline resistance

Strain	MRSA/MSSA	CC	*mepA*		*mepR*		*rpsJ*	
			nt change	AA change	nt change	AA change	nt change	AA change
497	MRSA	5	A861T C862T G1243C	Glu287Gly Leu288Phe Val415Leu	C185T	Pro62Leu	–	–
2028	MRSA	8	C86T A860G	Thr29Ile Glu287Gly	G236A	Arg79His	–	–
94159	MRSA	5	C86T	Thr29Ile	G73T	**Premature stop codon**	A170T	Lys57Met
74016	MRSA	5	C821T C862T	Ala274Val Leu288Phe	C211T	Leu71Phe	–	–
4261	MRSA	5	C86T A860G	Thr29Ile Glu287Gly	G100A C188A	Gly34Ser Thr63Asn	–	–
54081	MSSA	45	G130A G481A C862T	Gly44Arg Ala161Thr Leu288Phe	G96C	Glu32Asp	A170T	Lys57Met
74073	MSSA	6	A860G	Glu287Gly	72 bp deletion (90–161)	24 AA deletion (31–54)	A169C	Lys57Gln
14069	MSSA	1	T1328G	Val443Gly	G94T	**Premature stop codon**	–	–
34076	MSSA	45	C86T A860G	Thr29Ile Glu287Gly	G304T	**Premature stop codon**	A170T	Lys57Met
34204	MSSA	1	A860G A1209T	Glu287Gly Leu403Glu	12 bp insertion (62–73)	Addition of 4 AAs (24–27)	G178T	Asp60Tyr
44213	MSSA	30	G95T G512A	Ser32Ile Gly171Asp	Deletion of G (275)	**Premature stop codon**	C157A C161A	Arg53Ser Ala54Asp
34023	MSSA	1	C735T T1001G	Val234Gly	C172T	**Premature stop codon**	–	–

MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; CC, clonal complex; nt, nucleotide; AA, amino acid.
NOTE: Premature stop codon in MepR is indicated in bold.

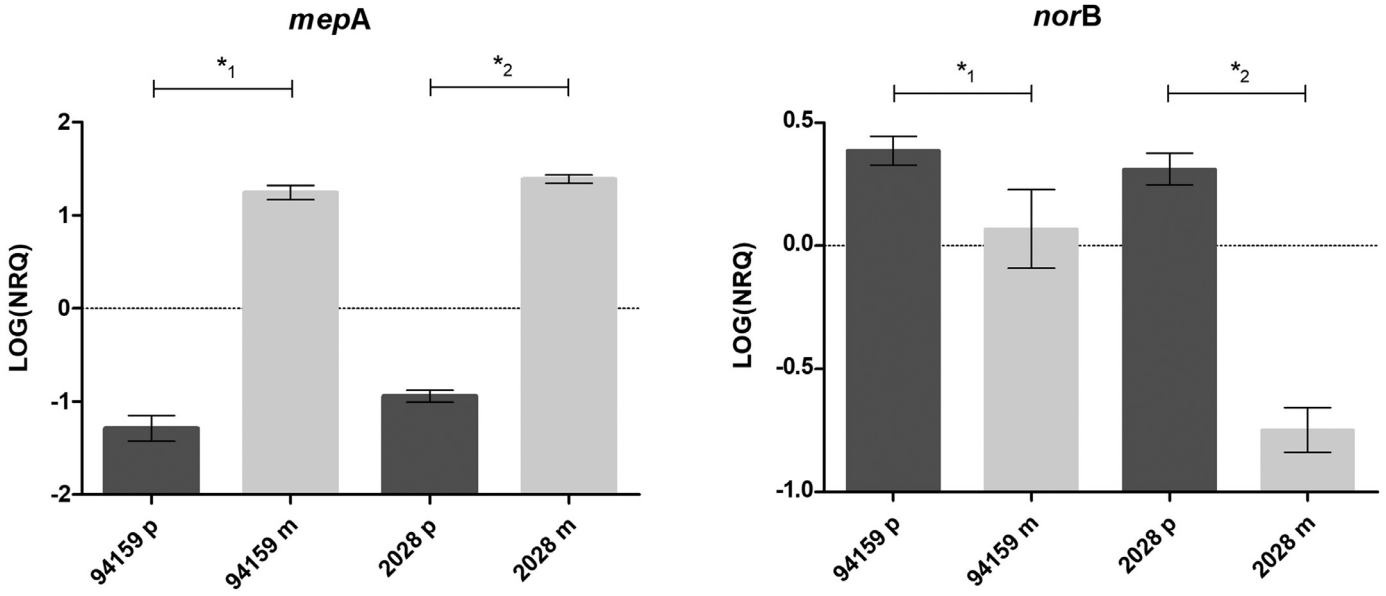

Fig. 1. Expression levels of the *mepA* and *norB* genes by reverse transcription quantitative real-time PCR (RT-qPCR). NRQ, normalised relative quantity. Bars represent the median and standard error of three determinations. Statistically significant differences (Student's test) are marked as follows: for *mepA*, *1, $P = 0.0009$, *2, $P < 0.0001$; and for *norB*, *1, $P = 0.1093$, *2, $P = 0.0007$.

Remarkably, a prophage of the Sa3*int* type carrying *sak* and *scn* genes (encoding proteins of the immune evasion cluster) was detected in 94159p but not in its derived mutant. In addition, 94159m harboured a copy of IS*256* inserted into the promoter region of the *walR* gene.

4. Discussion

It is well known that *S. aureus* has the ability to develop decreased susceptibility to various antibiotics in the presence of in vitro and in vivo selective pressure [7,29,30]. However, information regarding the molecular mechanisms involved in the emergence or selection of clinical *S. aureus* isolates resistant to TGC treatment is scarce. In this study, 12 TRSAm were obtained (5 MRSA and 7 MSSA). Isolates included in this work belonged to seven different genotypic lineages clustered in six clonal complexes, with a predominance of CC5, probably reflecting the prevalence of this clonal complex in our region during the time of sampling [31,32].

Development of antibiotic resistance has been associated with a reduction in bacterial fitness. By contrast, the results of this work suggest that the acquisition of TGC resistance in different *S. aureus* strains is not linked to a significant fitness cost. These results are in accordance with those described by Dabul et al., although in that study bacterial fitness was evaluated only through the determination of growth rate [9].

Resistance to TGC in bacterial isolates of different genera has been generally associated with overexpression of efflux pumps. This mechanism has been documented in some Gram-negative

species such as *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Salmonella enterica* and *Serratia marcescens* [33]. In addition, *S. aureus* efflux pumps have been associated with resistance to different antimicrobial agents and the selection of drug-resistant strains [34].

All 12 TRSAm showed an increase in their efflux activity as assessed by determination of the TGC MIC in the presence and absence of the efflux pump inhibitor RS.

The contribution of the MepA efflux pump to reduced susceptibility to TGC in *S. aureus* mutants obtained in vitro has been previously demonstrated [7,9]. Moreover, MepA has also been linked to decreased susceptibility to fluoroquinolones, biocide compounds and dyes [7]; however, the mutants obtained herein did not significantly modify ciprofloxacin MIC values.

Schindler et al. predicted the secondary structure of MepA and reported amino acid substitutions in the carboxy-terminal half of MepA, near the cytoplasmic face of the protein, that increased the efflux capacity [35]. In our study, a total of 12 different amino acid substitutions were detected, with a predominance in the transmembrane region (Thr29Ile, Ser32Ile, Ala161Thr, Gly171Asp, Glu287Gly, Leu288Phe and Leu403Glu). The remaining five substitutions were located in the cytoplasmic (2/12) and extracytoplasmic (3/12) loops. Except for MepA Ala161Thr mutation, the substitutions found in this work were not reported by Schindler et al., which may be related to the different substrates used during the mutant selection.

Furthermore, a variety of amino acid changes in the MepR protein were predicted by comparative analysis of *mepR* sequences: six substitutions (at positions 32, 34, 62, 63, 71 and 79); one deletion of 24 amino acids; one insertion of four amino acids; as well as the introduction of nonsense mutations (in 5 strains) leading premature stop codons. Schindler et al. found that Ala103Val is the most common substitution affecting MepR repressor function in clinical isolates [36]. Neither this mutation or any other described by these authors were found in our work.

Other studies analysed *mepA*/*R* sequences for TGC-resistant *S. aureus* selected in vitro with increasing TGC concentrations and detected different mutations than those found herein [7,9] even when the same drug was used for mutant selection. Except for the Val415 mutation described by Dabul et al. in MepA in separate experiments using a single MRSA ST5 isolate, no amino acid substitution position coincides with those detected in our work [9].

All of these findings reinforce that mutations occurring in the *mepA*/*R* genes owing to selective pressure exerted by TGC would not be associated with a specific type of modification but that these genes can be affected by a diverse spectrum of alterations. Expression of *mepA* analysed by RT-qPCR was found to be significantly increased in two TRSAm (ST100 and ST239) that showed different mutations in *mepA* and *mepR*. Overexpression of *mepA* has been previously observed by other authors in TGC-resistant mutants [7,9].

It has been previously shown that MepR exerts its repressive action on the MepA efflux pump by specific binding on the *mepA* gene, blocking transcription. In our work, overexpression of *mepA* was observed beyond the type of mutation found in MepR, in accordance with two independent studies [36].

The secondary structure of MepR, represented by six α-helices and a two-stranded antiparallel β hairpin, was determined by Kumaraswami et al. [37]. Considering this structure, most of the amino acid substitutions in MepR detected in TRSAm (4/6) were located in the DNA-binding domain (composed of helices α3, α4, β1 and β2). In this way, mutations in this domain might alter the MepR-mediated repression of *mepA* leading to deregulation of *mepA* expression. In addition, mutations found in MepA may contribute to increased efflux activity by directly affecting pump functionality.

Despite the NorB efflux pump conferring resistance to tetracyclines among other antimicrobial agents [6], no nucleotide alterations were found in *norB* and its expression was significantly decreased in 2028m. A similar trend of lower *norB* expression was also observed in 94159m.

Mutations in *rpsJ*, which encodes the 30S ribosomal protein S10, have been associated with decreased susceptibility to TGC in clinical *S. aureus* isolates and in vitro-selected mutants [4,10,11,38,39]. It has been suggested that this mutation could alter the ribosome structure affecting the TGC binding site with a consequent decrease in TGC susceptibility [40]. Six of the twelve TRSAm described herein harboured mutations in *rpsJ* leading to changes in the amino acid at position 57 in four mutants in accordance with previous reports [4,10,39].

On the other hand, 2/12 mutants have also modified their susceptibility to other antibiotics [12]. Modifications in β-lactam and vancomycin susceptibilities in 94159m (which became VISA phenotype) could be related to the insertion of IS*256* in the promoter region of *walR* as previously reported [41]. Also, the same mutant lost the Sa3 prophage carrying the virulence genes *sak* and *scn*. These findings highlight the impact of antibiotic selective pressure on gene mobilisation (or rearrangement) and its possible consequences not only in the resistance profile but also in virulence.

We have not found a specific genetic change known to be associated with the modification of oxacillin susceptibility observed for 497m/497p. This mutant has a substitution in the *rpoA* gene (Supplementary Table S4) encoding the RNA polymerase α subunit. Various mutations in *rpoB* and *rpoC* (encoding β and β' subunits of RNA polymerase, respectively) were related to changes in MICs to β-lactams and vancomycin among other antibiotics [42–45]. In this sense, mutation in *rpoA* should not be underestimated and further studies must be conducted in order to elucidate its role.

5. Conclusion

Increased activity of the MepA efflux pump associated with mutations in the *mepA* gene as well as its regulatory gene *mepR* is the main mechanism of TGC resistance detected in in vitro-selected mutants of *S. aureus.* Mutations in *rpsJ* and *rpsN* genes (encoding 30S ribosomal proteins) might also contribute to TGC resistance. Finally, it is important to highlight that in vitro TGC pressure could select resistant variants harbouring mutations in *mepA, mepR* and *rpsJ* in 12 different *S. aureus* strains (7 MSSA and 5 MRSA) belonging to diverse genetic lineages that are also distributed worldwide (CC5, CC8, CC30, CC45, CC1 and CC6). Our findings demonstrate that this acquired mechanism of TGC resistance is not restricted to a single type of genotypic background and therefore different lineages might have the same plasticity to develop TGC resistance.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2021.106304.

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