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Supplementary Information

Heritable Nanosilver Resistance in Priority Pathogen: A Unique Genetic Adaptation and Comparison with Ionic Silver and Antibiotic

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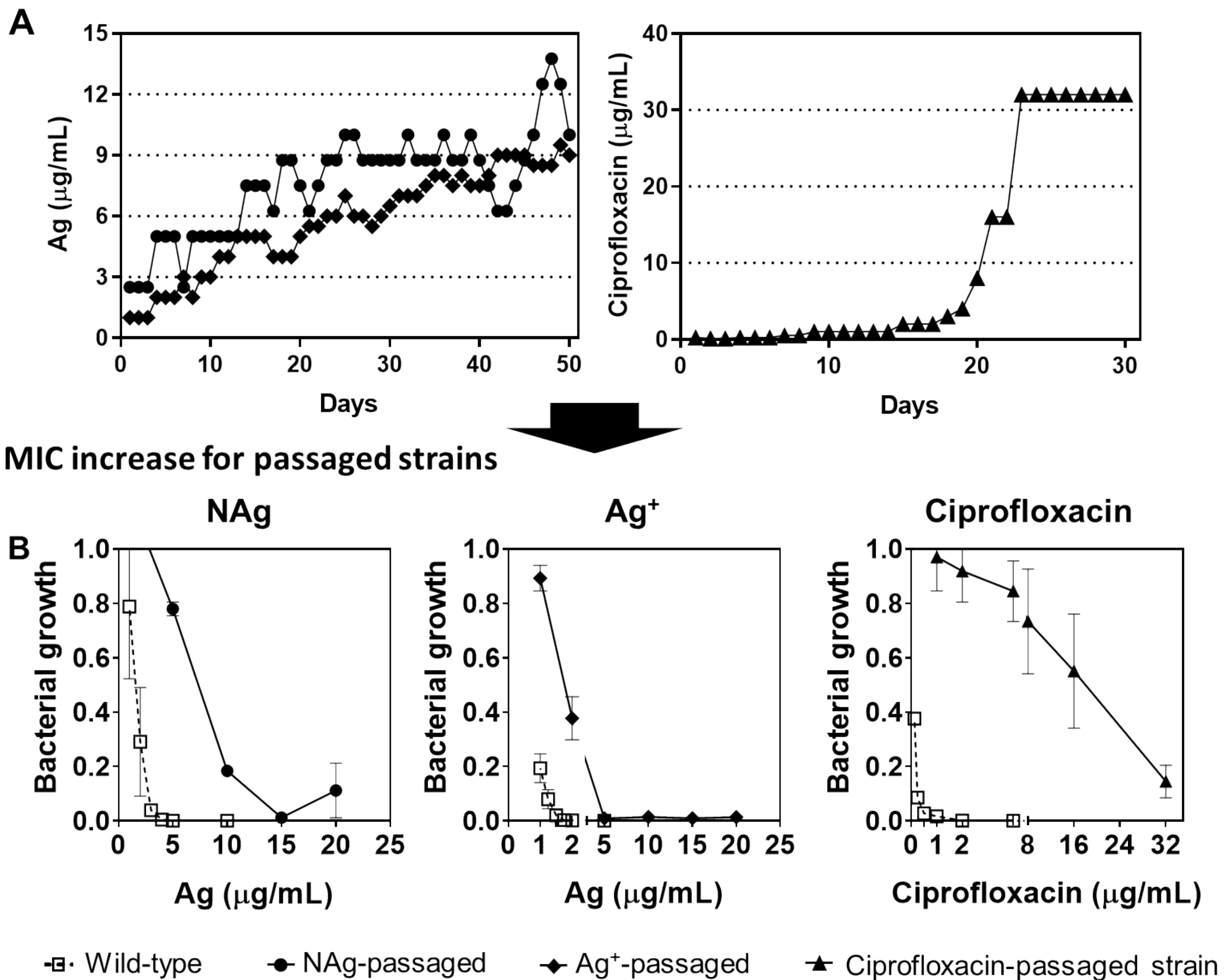
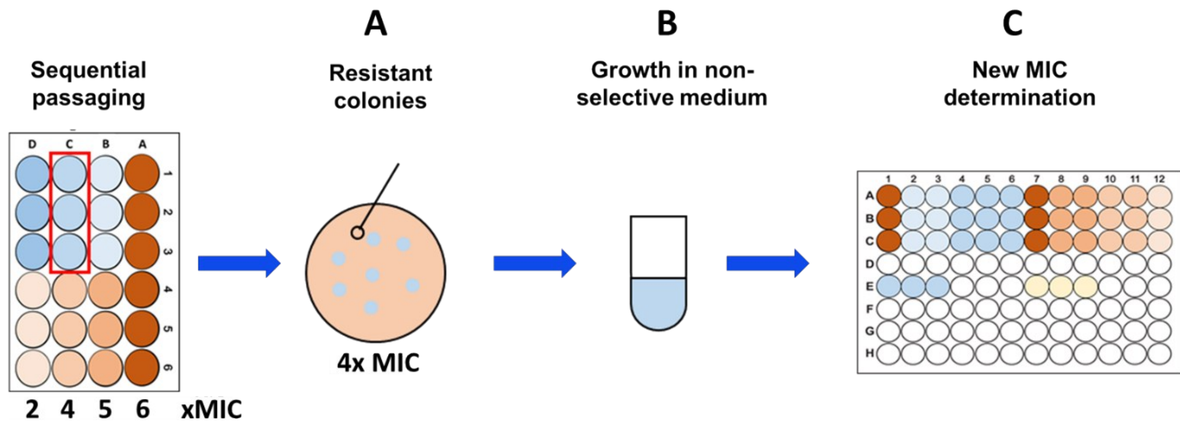


Figure S1. Biological replicates: The development of resistance to NAg, ionic silver and the antibiotic ciprofloxacin in *S. aureus*. (**A**) Continuous exposure *via* passaging (sub-culturing) of *S. aureus* in the presence of progressively increasing concentration of NAg (for 50 d), ionic silver (50 d) and ciprofloxacin (30 d), starting at sub-MIC levels of the respective antimicrobials. (**B**) Post-exposure changes in the MIC of the antimicrobials. The extent of growth (24 h, 37ºC, relative to the no antimicrobial control) of the passaged *S. aureus* was assessed in the presence of NAg (1 to 20 µg Ag/mL, solid profile), ionic silver (1 to 20 µg Ag/mL) and ciprofloxacin (0.13 to 32 µg/mL) and compared to those of the wild-type (dotted profiles). For the MIC work, the data point shown is the average of three biological replicates (experiments with independent bacterial inocula; from three isolates in the case of the passaged strains and different antimicrobial preparations) and each biological replicate with three technical replicates, with error bars representing the standard error of measurement (SEM).



Scheme S1. Steps for the determination of MIC increase after the prolonged antimicrobial exposures (passaging) of *S. aureus*. (**A**) Growth of the passaged strain on agar containing the respective antimicrobial, (**B**) culturing of the grown colonies in antimicrobial-free liquid medium (3 d sub-culturing), (**C**) dose-response growth study for 'new' MIC determination of the antimicrobials.

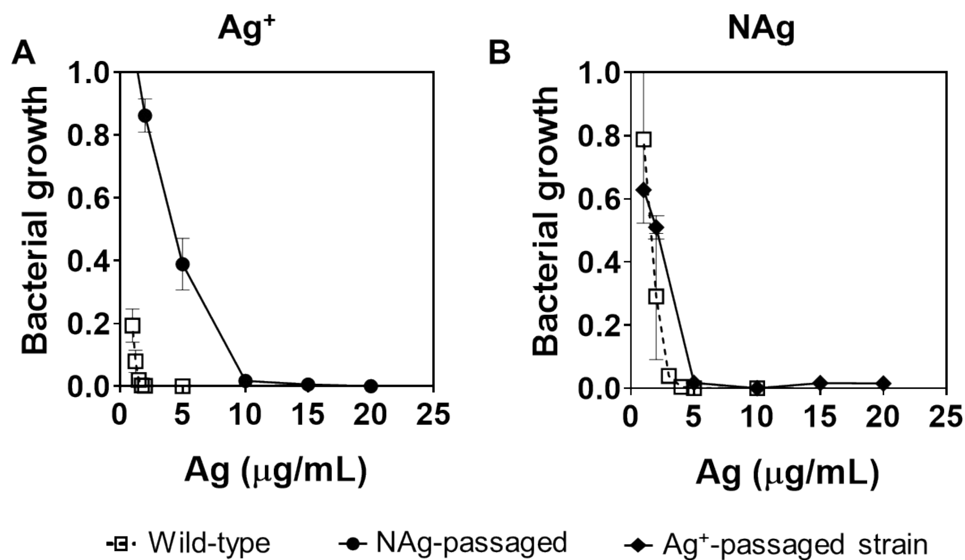


Figure S2. Biological replicates: NAg/ionic silver cross-resistance in *S. aureus*. (**A**) The extent of growth (24 h, 37ºC, relative to the no antimicrobial control) of the NAg-passaged strain in the presence of ionic silver (1 to 20 µg Ag/mL, solid profile) and (**B**) the ionic silver-passaged strain in the presence of NAg (1 to 20 µg Ag/mL, solid profile) compared to the respective wild-type (dotted) profiles. Each data point is the average of three biological replicates, each with three technical replicates, with the error bars representing the standard error of measurement (SEM).

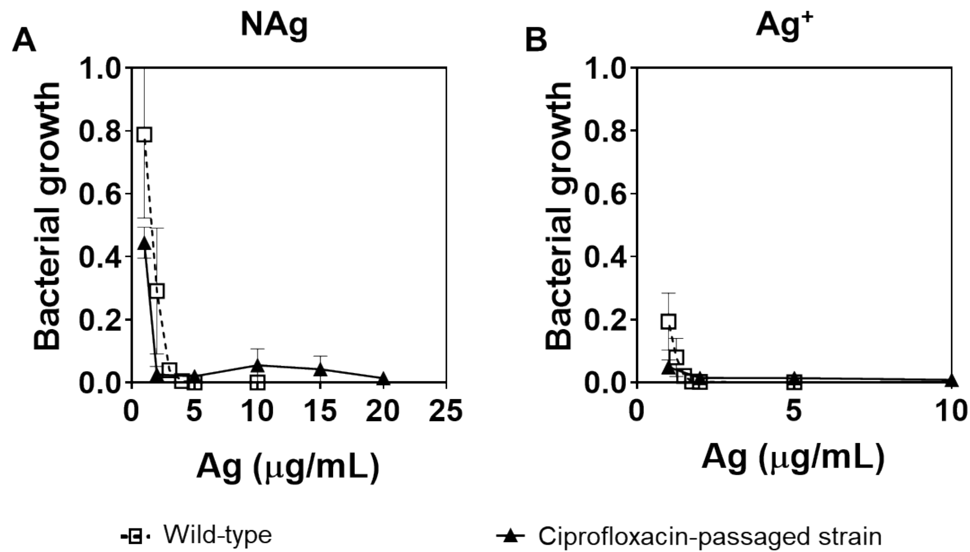


Figure S3. The extent of growth (24 h, 37ºC, relative to the no antimicrobial control) of the ciprofloxacin-passaged *S. aureus* in the presence of (**A**) NAg (1 to 20 μg Ag/mL, solid profile) and (**B**) ionic silver (1 to 10 μg Ag/mL) were compared to the respective wild-type (dotted) profiles. Each data point is the average of three biological replicates, each with three technical replicates, with the error bars representing the standard error of measurement (SEM).

Silver-induced genomic mutations

The study detected mutations in the NAg- and ionic silver-resistant *S. aureus* using whole genome sequencing. The bacterium developed different gene mutations in response to the NAg 50 d exposure when compared to ionic silver, while there were also mutations in functionally related proteins in response to both the nanoparticle and ionic forms of silver. **Table S1** describes the additional mutations that were detected in the silver resistant mutants. These mutations, unlike those that are presented in the main text, were found in only one of the biological replicates of the prolonged silver exposures. All of these mutations however, like those that are presented earlier, have also been confirmed through PCR amplification and sequencing of the genes (in different bacterial isolates, see Experimental Sections).

Table S1. Additional point mutations detected in NAg- and ionic silver-resistant *S. aureus* genome

Nucleotide change in gene							Amino acid change in protein	Hypothesized defence mechanisms to silver toxicity
Gene locus	[a]Position in genome	Mutation type	Reference	Variation	*f* (%)	Gene annotation		
NAg-resistant strain								
KQ76_06080	1,232,347	SNV	G	T	100	UDP pyrophosphate synthase *uppS*	Asp195Tyr	The UppS protein functions as a catalyst for undecaprenyl dipyrophosphate (UPP) biosynthesis, a precursor for lipid II, an important component in the second-half of peptidoglycan synthesis process.[1] The mutation may decrease the UPP levels and lead to less lipid II synthesis, consequently elevating the intermediates from the first-half of the peptidoglycan synthesis process.[2] A study has shown that reduced UppS levels was observed to increase susceptibility to β-lactam antibiotics (competitively bind with lipid II), as also observed in this study.[3]
KQ76_10725	2,102,445	SNV	G	A	100	Phosphoserine phosphatase *rsbU*	Asp134Asn	The RsbU protein activated σ^B, a protein that influences many genes to cope with environmental (e.g. peroxides, UV) and antibacterial stress.[4] The mutation may alter levels of σ^B to allow cells to survive in the presence of NAg.
Ionic silver-resistant strain								
KQ76_01810	386,992	SNV	G	T	100	*L*-cystine uptake protein *tcyP*	Met357Ile	Together with the TcyA protein, the TcyP protein facilitates *L*-cystine uptake to cytoplasm for the synthesis of *L*-cysteine,[5] an essential compound to fight off silver-generated ROS.[6–8] Due to the potential for cysteine-mediated oxidative stress, the mutation could be to reduce the increase in cellular cysteine pools already reported during ionic silver exposure.[9]
KQ76_03830	788,889	SNV	A	G	100	Thioredoxin reductase *trxB*	Tyr133Cys	The enzyme thioredoxin reductase catalyzes the reduction of thioredoxin to its (reduced) active form to scavenge ROS,[10] generated by silver exposure.[11] The mutation is thought to provide additional cysteine (a change of Tyr to Cys) in the thioredoxin reductase active site,[8] possibly to cope with the Ag^+ binding to the 'original' cysteine in the active site.
KQ76_00825	1,700,650	SNV	G	C	100	Adenine phosphoribosyltransferase *apt*	Met1Ile	The Apt protein catalyzes the formation of adenine (purine nucleotide) from adenosine monophosphate (AMP).[12] The mutation results in the absence of methionine (Met) in initiation codon that could lead to a very low degree of the Apt protein synthesis,[13] which in turn may alter the energy pools available to support growth in the presence of NAg.[14]

KQ76_06710	1,354,358	SNV	T	G	100	Phosphatidylglycerol lysyltransferase *mprF*	Cys380Trp	The enzyme catalyzes the transfer of a lysyl group from L-lysyl-tRNA(Lys) to phosphatidylglycerol to generate the positively-charged lysilphosphatidylgycerol in *S. aureus* cell membrane.[15] The mutation could lead to an increased net positive charge of the cell envelope,[16,17] thereby repelling the Ag^+ ion.[18] A mutation to this gene was found to associate with a reduced resistance to the β-lactam oxacillin and methicillin.[19]
KQ76_10800	2,116,579	SNV	T	C	100	RNA helicase *cshA*	Ser451Pro	The protein CshA is an ATP-dependent RNA helicase, an enzyme that is involved in RNA metabolism, including in RNA splicing and translation initiation.[20] The mutation is thought to reduce energy usage for RNA metabolism.[20]
KQ76_04420	903,745	SNV	C	T	100	CoA-disulfide reductase *cdr*	Gln141*	The enzyme catalyzes the formation of CoA-disulfide from CoA (coenzyme A) with the expense of the cofactor NAD^+ or $NADP^+$. The process is key in the regulation of thiol (sulphur) metabolism to maintain cellular redox balance.[21]

[a]In *S. aureus* ATCC 25923

SNV (single nucleotide variation) denotes substitution-type change of a single nucleotide at a specific position in the genome, *f* = frequency of the nucleotide changes occurring in a bacterial isolate, * = stop codon.

Note: Our follow-up RNAseq work supports the likely minor role of several of these gene mutations for NAg resistance (*e.g.* the *uppS* gene, involved in cell wall synthesis of *S. aureus*) and ionic silver resistance (*e.g.* the *tcyP* gene, involved in cysteine metabolism) (unpublished data).

Table S2. Primers used for confirmation of the gene mutation

	Forward primer (5'→ 3')	**Reverse primer (5' → 3')**	**Annealing Tm (°C)**	**Amplicon size (bp)**
purR	CGCAAGTGGTGGTGTTACG	CACCATTGATAGAGCCACCA	50	452
tcyA	GCAGGGCGTTTTGATGTAAT	AGAAACATCTTGACCAAACCA	50	500
cymR	GTTTTTGCGCAGGTGGTT	AAAAAGAGGGGCAAGGATGT	50	249
uppS	TTCCACTGAAAATTGGTCAAGA	TCGTCAAAGTCAGGCCATAA	50	468
rsbU	CAATGCTTAAAACAGATATTCCACA	TCAGTCACACCATCCGTTAAA	50	505
tcyP	GCAACGCCGAATGAACTAAT	GCATTCACATCACGCTCAAG	50	251
trxB	GTATTCCAGGCGGTCAAATG	TGAGTCACCACCACCGATAA	52	356
apT	TGCTGCTTCAATCGTACCAC	TCGTGAGCAATTTAGGAGGAA	50	417
mprF	TTCATTCCGGCTAAAGATGTG	TGATAATCGAATAACCACGCAAT	50	605
cshA	GTCTGCTTCCACCGCTACTC	TTCGTCCACCACATCGTAAA	50	387
cdr	CCCTGCACCTACAACCAATA	TCATTGGCGAAGTTGTTGAA	52	329

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