In vitro colistin susceptibility of pandrug-resistant *Acinetobacterbaumannii* is restored in the presence of selenium nanoparticles

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Abstract

Aims: To investigate the synergistic activity of colistin and selenium nanoparticles (SeNPs) against pandrug-resistant (PDR) *Acinetobacterbaumannii*.

Methods and results: Chequerboard and time-kill assays were employed to explore the potential synergistic interactions between colistin and SeNPs against *A. baumannii* isolates (8), previously determined as colistin-resistant (MIC range 16–256 μg ml^{-1}). Also, whole-genome sequencing (WGS) and gene expression analyses were used to elucidate the mechanisms of colistin resistance. Exceptionally strong synergistic activity (FICI range 0.004–0.035) of colistin and SeNPs against colistin-resistant isolates was revealed. Colistin (0.5 or 1 μg ml^{-1}) used in combination with SeNPs (0.5 μg ml^{-1}) was able to reduce initial inoculum during the first 4 h of incubation, in contrast to colistin (0.5, 1 or 2 μg ml^{-1}) alone.

Conclusions: These findings propose colistin/SeNPs combination as a new option to fight PDR *A. baumannii*, the therapeutic possibilities of which should be proved in future *in vivo* studies.

Significance and impact of study: Here we present the first evidence of synergy between colistin and selenium compounds against bacteria in general. Also, WGS and gene expression analyses provide some new insights into *A. baumannii* colistin resistance mechanisms.

Keywords: *Acinetobacterbaumannii*; Colistin; Synergy; Selenium nanoparticles; WGS; Resistance mechanisms

Introduction

Acinetobacterbaumannii causes difficult-to-treat nosocomial infections, frequently associated with high mortality rates. The most common clinical manifestations are ventilator-associated pneumonia and bacteraemia (catheter-related or secondary), followed by deep wound infections, urinary tract infections and meningitis (Wong *et al*. 2017). These infections are most often very hard to treat, due to the limited number of effective clinically available antibiotics. Of particular concern is the widespread emergence of carbapenem-resistant *A. baumannii* (CRAB) strains, lacking beneficial therapeutic options in frequent situations (Kempf and Rolain 2012). For this reason, the World Health Organization (WHO) and, more recently, the US Centers for Disease Control and Prevention (CDC) have listed CRAB as a pathogen that urgently requires the development of new therapeutic strategies (Tacconelli *et al*. 2018; CDC 2019).

In most cases, colistin is the only effective choice for the treatment of infections caused by CRAB. This 'last resort' antibiotic appears to be highly efficient and reasonably safe when used in appropriate dosage, so that the plasma concentration is maintained at approximately 2 μg ml^{-1} (Garnacho-Montero and Timsit 2019). However, colistin-resistant *A. baumannii* strains are increasingly emerging in hospital settings worldwide (Nowak *et al*. 2017). Considering that these strains are usually pandrug-resistant (PDR), i.e. non-susceptible to all clinically available antibiotics, there is an unambiguous need for the discoverof new therapeutic solutions.

Selenium nanoparticles (SeNPs) are ultrafine particles of stabilized elemental selenium (Se^0) with well-established antimicrobial properties (Hosnedlova *et al*. 2018). The most widely accepted mechanism of their antibacterial action is the induction of reactive oxygen species generation (Geoffrion *et al*. 2020). Recently, it has been demonstrated that ebselen, an organoselenium compound, exhibits potent bactericidal activity against *A. baumannii* by inhibiting thioredoxin reductase and by depleting the total available glutathione cellular pool (Dong *et al*. 2020). The latter components are involved in multiple cellular processes

including antioxidant defence, DNA synthesis and repair, and protein folding (Du *et al*. 2012; Lu and Holmgren 2014), thus suggesting the possibility that selenium could act in synergy with certain antibiotics. Moreover, nanosized elemental selenium offers higher bioavailability and much lower toxicity compared to the organic and inorganic, oxidized forms of selenium (Hosnedlova *et al*. 2018).

In this study, we identified eight colistin-resistant isolates of *A. baumannii* for which we performed whole-genome sequencing (WGS) and quantitative reverse transcription PCR (RT-PCR) to elucidate the molecular basis of colistin resistance. Then we investigated whether the addition of SeNPs at low concentrations could restore colistin susceptibility in these strains *in vitro*. In addition, we tested the effect of colistin/SeNPs combination against several colistin-susceptible strains.

Materials and methods

Bacterial strains

Eight colistin-resistant and three colistin-susceptible *A. baumannii* strains were used in this study (Table 1). The isolates were collected between late autumn 2017 and early spring 2018 from four health facilities in Belgrade, Serbia (Clinical Hospital Centre DrDragišaMišovićDedinje, Clinic for Gynecology and Obstetrics of University Clinical Centre of Serbia, Clinical Hospital Centre Zvezdara and Beo-lab outpatient laboratory). All isolates were initially identified at these health facilities as carbapenem-resistant *Acinetobacter* spp. using VITEK® 2 identification (GN ID) and susceptibility (AST-N240) cards (bioMérieux, Marcy-l'Étoile, France). Species-level identification was later obtained by 16S rRNA gene sequencing.

WGS analysis of colistin-resistant isolates

Broth microdilution method, performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2015), was used for determination of susceptibility to

colistin(Sigma-Aldrich, St. Louis, MO). Colistin-resistant isolates were subsequently genotyped by Pulsed-field gel electrophoresis (PFGE) as described(Kojic *et al*. 2005) and their genomes were sequenced using Illumina HiSeq by MicrobesNG service (IMI-School of Biosciences, University of Birmingham, Birmingham, UK). Following the quality control check using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), sequenced genomes were screened for the presence of antimicrobial resistance genes using ResFinder 4.1 tool, and multilocus sequence typing (MLST) analysis was performed using MLST 2.0 (Pasteur scheme), both services available at the Center for Genomic Epidemiology (www.genomicepidemiology.org). Potential colistin resistance-conferring mutations were then identified by comparative analysis of the *lpxACD* and *pmrCAB* operon sequences with the corresponding genes of colistin-susceptible *A. baumannii* ATCC 19606 reference strain (GenBank GCA_002811175.1) using DNA Strider. Also, the presence of insertion sequence (IS) elements upstream of the *eptA* gene was analysed by IS Finder (https://isfinder.biotoul.fr/). In addition, pan-genome analyses were carried out to further elucidate genomic relatedness of sequenced strains using Prokka (v1.13), Roary (v3.13.0) and Phandango services, as described previously (Jovčić *et al*. 2020). Gene was considered as a part of core genome if it appeared in >99% strains. Accessory genes were subdivided into shell genes (present in 15–95% strains) and cloud genes (present in less than 15% strains).

Draft genome sequences of eight colistin-resistant *A. baumannii* isolates have been deposited in the NCBI GenBank database under accession numbers JAFFOV000000000–JAFFPC000000000.

Colistin resistance-associated genes expression analysis

Colistin-resistant strains were analysed for the *lpxA, lpxC, lpxD, pmrC, pmrA* and *pmrB* genes expression using quantitative real-time RT-PCR. The isolates were incubated in Mueller-Hinton broth at 37°C overnight with shaking, then diluted in fresh broth and grown to OD_{600} of 0.5. The RNA isolation, DNase I treatment and reverse transcription were performed as previously described (Novović *et al.* 2018). Quantitative RT-PCR was carried out on a 7500 Real Time PCR

System thermocycler (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA), using the KAPA SYBR Fast qPCR Kit (KAPA Biosystems, Boston, MA) and primers described in previous studies (Cafiso *et al*. 2019; Adams *et al.* 2009; Coyne *et al*. 2010). Normalization was done against the *rpoB* gene using the *ΔΔCT* method (relative) (Livak and Schmittgen 2001). The obtained values were then normalized against the expression levels of the corresponding genes measured in colistin-susceptible *A. baumannii* ATCC 19606 strain.

Selenium nanoparticles (SeNPs) synthesis and antimicrobial activity evaluation

SeNPs were synthesized by chemical reduction of sodium selenite (Sigma-Aldrich), using ascorbic acid (reducing agent; VWR BDH Prolabo, Leuven, Belgium) and BSA (stabilizer), as described previously (Filipović *et al*. 2021). Initially, 10 and 5 ml of ascorbic acid (0.125 mol l^{-1}) and BSA (0.87% w/w) distilled water solutions, respectively, were prepared and mixed together. Then, the mixture was placed on a magnetic stirrer (1,500 rpm) and supplemented with 0.02 mol l^{-1} distilled water solution of sodium selenite, gradually (12.5 ml in total), until the red-orange colour was achieved. Final mixture was prepared in a way so that the mass ratio between BSA and sodium selenite was adjusted to 1:1. All reaction vessels were covered using aluminium foil during the synthesis procedure, to prevent crystallization and photo-oxidation of SeNPs. Broth-microdilution method was used to determine MICs of synthesized SeNPs against *A. baumannii* strains (CLSI, 2015). The tested concentration range (32-512 μg ml^{-1}) of SeNPs was prepared by diluting in Mueller-Hinton broth, which was subsequently used as a growth medium.

Chequerboard assays

The antibacterial activity of colistin/SeNPs combination against both colistin-resistant and colistin-susceptible *A. baumannii* strains was assessed by chequerboard assays (Stein *et al*. 2015). Colistin was applied over the concentration ranges of 0.25–256 and 0.004–4 μg ml^{-1} for colistin-resistant and colistin-susceptible strains, respectively. The SeNPs range tested was 0.125–8 μg ml^{-1} for all strains. Both compounds were diluted in Mueller-Hinton broth, which

was further used as a growth medium. The interaction types were determined based on the calculated fractional inhibitory concentration indices (FICIs). FICIs were interpreted as follows: ≤0.5, synergy; >4.0, antagonism; >0.5–4.0, no interaction.

Time-kill assays

The growth kinetics of colistin-resistant *A. baumannii* isolates treated with colistin/SeNPs or colistin alone was evaluated using time-kill assays, performed according to CLSI guidelines (CLSI, 1999). Colistin was tested at 0.5, 1 and 2 μg ml^{-1} (selected based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria for susceptibility to colistin) alone, and at 0.5 and 1 μg ml^{-1} in combination with 0.5 μg ml^{-1} of SeNPs. Fresh overnight cultures were diluted in 5 ml of Mueller-Hinton broth to ~10^5 cfu ml^{-1} and incubated at 37°C. Viable cell counts were performed at 0, 4, 8 and 24 h, following the subcultivation of serial dilutions on Mueller-Hinton agar plates at 37°C for 24 h, by using drop plate technique.

Statistical analysis

All experiments were repeated in at least three independent replicates. Student's *t* test was used to compare differences in results of quantitative RT-PCR obtained for colistin-resistant *A. baumannii* isolates and colistin-susceptible *A. baumannii* ATCC 19606. The results are presented as mean values ± standard deviations (SD). A *P* value <0.05 was considered significant.

Results

Genotyping, pan-genome and resistome analyses of colistin-resistant isolates

Colistin-resistant *A. baumannii* isolates (*n* = 8) originating from three different health facilities were identified in this study. Obtained colistin MIC values against these strains ranged from 16 to 256 μg ml^{-1} (Table 1). Seven of them were hospital isolates, whereas one isolate (B103)

originated from an outpatient laboratory. Six of the hospital isolates (D650, D749, D811, D1002, D441, D1010) were isolated in the Clinical Hospital Centre DrDragišaMišovićDedinje.

The PFGE analysis revealed a high level of genetic relatedness among seven colistin-resistant isolates, still each isolate had a unique pattern of ApaI restriction fragments (Fig. S1). On the other hand, considerably different genetic profile was displayed by the isolate D1010. Subsequently, it was determined by MLST analysis (Pasteur) that the isolate D1010 belongs to distinct ST492 sequence type, whereas the remaining seven isolates were assigned to ST2 sequence type.

A total of 4164 genes were detected by the pan-genome analyses, nearly 79% (i.e. 3278 genes) of which comprised the core genome, thus confirming the high degree of phylogenetic relatedness among the isolates. The rest of the pan-genome, i.e. the accessory genome, was composed of 643 shell genes and 243 cloud genes. Isolates sharing the same accessory genes were clustered together (Fig. S2). According to this, isolates D650, D749, D811 and D1002 contained accessory genome structure highly similar to that of the B103 strain, isolated in an outpatient setting, suggesting that some of the related hospital colistin-resistant isolates may have been introduced into the community. Slightly different was the accessory genome of D441 and G1812 isolates, in contrast to the isolate D1010 for which notably different genome structure was observed.(Oikonomou *et al.* 2015; ThiKhanhNhu *et al*. 2016; Nurtop *et al.* 2019; Jovcic *et al*. 2021). Nevertheless, the additional mutations that D1010 contained (R263L, H266L) were located in the region corresponding to the histidine kinase domain of PmrB (ThiKhanhNhu *et al*. 2016), which is frequently altered in colistin-resistant isolates (Cafiso *et al*. 2019; Nurtop *et al.* 2019; Jovcic *et al*. 2021), therefore, it is reasonable to assume that they could have contributed to the colistin resistance. Also, the mutation T68N, to the best of our knowledge, has not been described so far and its involvement in colistin resistance needs yet to be investigated. The particular substitutions that we identified in LpxC, LpxD and PmrC were also previously detected. Single alterations contained by LpxC and LpxD were described in both

colistin-resistant and colistin-susceptible strains (Oikonomou *et al.* 2015; Nurtop *et al.* 2019; Jovcic *et al*. 2021), making them unlikely to be associated with the colistin resistance, however, the ones present in PmrC, were only described in colistin-resistant strains before (Jovcic *et al*. 2021). Transcriptional analysis further revealed that the genes encoding LpxC and LpxD proteins, as well as the *lpxA*, were significantly downregulated in tested isolates, which may have led to the decreased production of the LOS, possibly contributing to the colistin resistance (Moffatt *et al*. 2010; Jovcic *et al*. 2021). The expression of *pmrC*, *pmrA* and *pmrB* was also significantly inhibited in all tested isolates, which was, on the other hand, quite unexpected considering that usually these genes are highly overexpressed in the colistin-resistant *A. baumannii* (Adams *et al*. 2009; Beceiro *et al*. 2011).

A steady progress in the field of nanomedicine has been made in recent years. There are now more than 100 products and procedures based on the use of nanocomponents approved by the US Food and Drug Administration (FDA), including recently developed mRNA-based COVID-19 vaccines (Farjadian *et al*. 2019; Tang *et al*. 2021). SeNPs are considered to be well-tolerable in animal hosts. Several *in vivo* studies revealed that signs of acute toxicity appear only when using high doses, i.e. above 20 mg kg^{-1}, and established LD_{50} ranging from 92.1 to 198.1 mg kg^{-1} (Wang *et al*. 2007; Zhang *et al*. 2008; Shakibaie *et al*. 2013). Symptoms of chronic toxicity were observed following the peroral admiistration of above 2 mg kg^{-1} SeNPs for 14 consecutive days (He *et al*. 2014). In this study we demonstrated that *in vitro* colistin susceptibility in *A. baumannii* can be restored if colistin is used in combination with only 0.5 µg ml^{-1} of SeNPs. Besides, the synergy was most probably not the result of mere combination of antimicrobial effects of interacting agents, since the synergistic concentration of SeNPs was much lower than its MIC values (128–256 µg ml^{-1}). It could have been, however, a consequence of some other type of activity exhibited by SeNPs that perhaps enabled colistin to circumvent the resistance mechanisms. Most importantly, seven PDR strains, against which there is currently no effective therapy in most of the healthcare systems worldwide, were made susceptible to this

otherwise highly efficient antibiotic, in *in vitro* conditions. Considering the high bioavailability of SeNPs (Zhang *et al*. 2008), the concentration of 0.5 μg ml^{-1} should be easily achieved in the plasma by the systemic administration of a subtoxic dose. Although the time-kill assays did not show an evident bactericidal effect and treated strains tended to regrow after the initial inhibition, the achieved growth delay as well as mild reduction in the final log_{10} cfu may well significantly influence the ultimate outcome of the hypothetical host-pathogen battle. As it is generally accepted, the clinical outcome of *A. baumannii* infection largely depends on the level of bacterial burden/span> (Wong *et al*. 2017), thus it is reasonable to assume that even the slight reduction in bacterial growth could provide a key advantage to the host immune system.

In summary, the results of this study are valuable for several reasons: (i) it is the first evidence of synergistic interaction between colistin and selenium compounds against bacteria in general; (ii) synergistic activity was achieved by low concentrations of interacting agents, which should be easily reached by the systemic administration *in vivo*; (iii) existing literature, as described above, suggests that the active concentration of SeNPs should be achieved *in vivo* without any toxic side effects; (iv) PDR *A. baumannii* strains, usually lacking effective therapy, were made susceptible to colistin, which is a readily available therapeutic option. We conclude that our findings constitute a good basis for future *in vivo* studies, which should be carried out to investigate the therapeutic possibilities of the combined use of colistin and SeNPs.

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Conflict of interests

No conflict of interest declared.

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Authors contribution statement

DušanUšjak: Conceptualization, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Katarina Novović:** Formal analysis, Investigation, Writing - Original Draft. **BrankicaFilipić:** Formal analysis, Investigation, Data Curation. **Milan Kojić:** Validation, Formal analysis, Funding acquisition, Writing - Review & Editing. **NenadFilipović:** Investigation, Resources. **Magdalena M. Stevanović:** Investigation, Resources. **Marina T. Milenković:** Validation, Resources, Writing - Review & Editing, Supervision, Project administration.

Data availability statement

Research data are not shared.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 PFGE profiles of colistin-resistant *A. baumannii* isolates obtained by *ApaI* digestion. M-λ concatemers (New England Biolabs); 1-B103; 2-D650; 3-D749; 4-D811; 5-D1002; 6-G1812; 7-D441; 8-D1010. The arrows indicate bands that are not present in all isolates

Fig. S2 Visualization of the pan-genome of colistin-resistant *A. baumannii* isolates based on the core and accessory genes showing phylogenetic relatedness of the isolates by blue (present) and white (absent) fragments. At the bottom of the figure the percentage of strains that have each gene is shown

Fig. S3 FICI ranges of each colistin/SeNPs synergistic combination against each colistin-resistant *A. baumannii* strain. Bounded boxes indicate the most synergistic combinations

Table S1 The results of antimicrobial susceptibility tests on selected *A. baumannii* strains

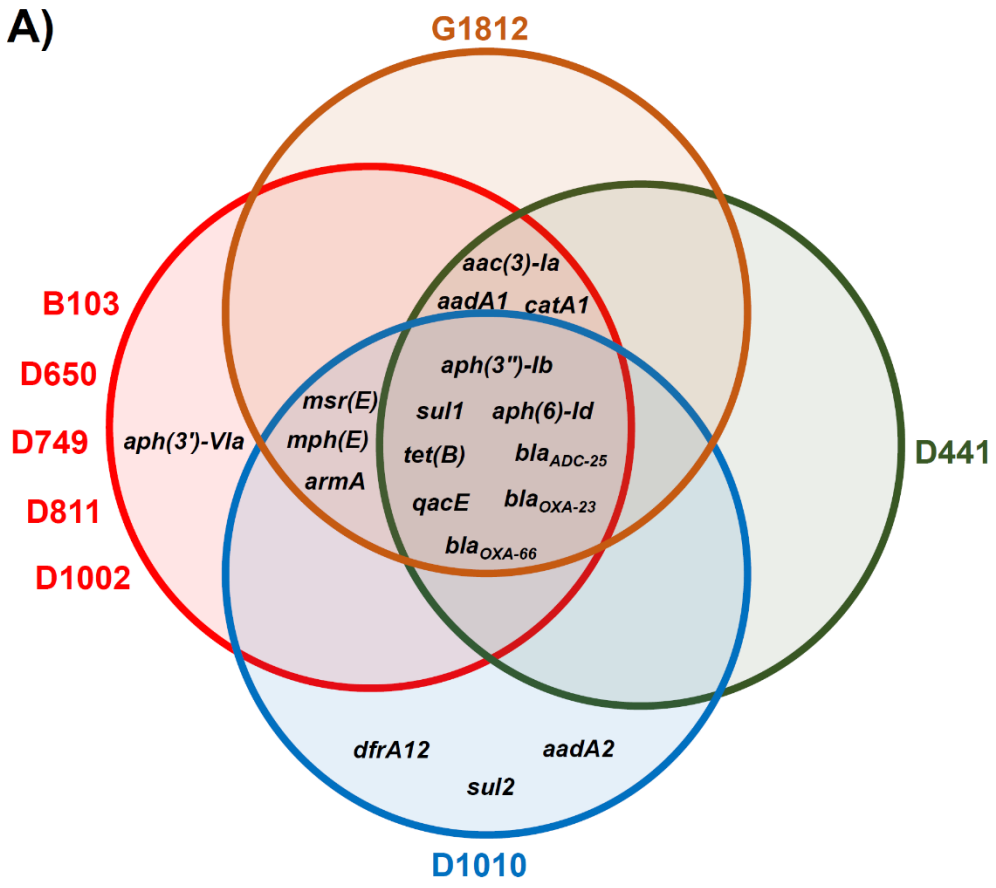
Figure and Table legends

Fig. 1 Colistin-resistant *A. baumannii* isolates: (A) resistomes and (B) amino acid alterations in colistin resistance-associated proteins, displayed as Venn diagrams

Fig. 2 Transcriptional analysis of colistin resistance-associated genes in colistin-resistant isolates and colistin-susceptible ATCC 19606 strain. (A) Relative expression of the *lpxA*, *lpxC* and *lpxD* genes. (B) Relative expression of the *pmrC*, *pmrA* and *pmrB* genes. (■) ATCC 19606, (■) B103, (■) D650, (■) D749, (■) D811, (■) D1002, (■) G1812, (■) D441, (■) D1010. Data are expressed as the mean ± SD. ***P* <0.01, ****P* <0.001

Fig. 3 Time-kill curves of colistin-resistant *A. baumannii* isolates exposed to colistin (CST) alone or colistin/SeNPs. (×) Control, (□) CST 2 μg ml^{-1}, (△) CST 1 μg ml^{-1}, (○) CST 0.5 μg ml^{-1}, (▲) CST 1 μg ml^{-1} + SeNPs 0.5 μg ml^{-1}, (●) CST 1 μg ml^{-1} + SeNPs 0.5 μg ml^{-1}. Data are expressed as the mean ± SD

Table 1 Origin and features of *A. baumannii* strains, and results of susceptibility (MIC) and synergy (FICI) analyses


A)
G1812
B103
D650
D749
D811
D1002
aph(3')-VIa
D441
D1010
aac(3)-Ia
aadA1
catA1
aph(3")-Ib
msr(E)
mph(E)
armA
sul1
aph(6)-Id
tet(B)
bla ADC-25
qacE
bla OXA-23
bla OXA-66
dfrA12
aadA2
sul2

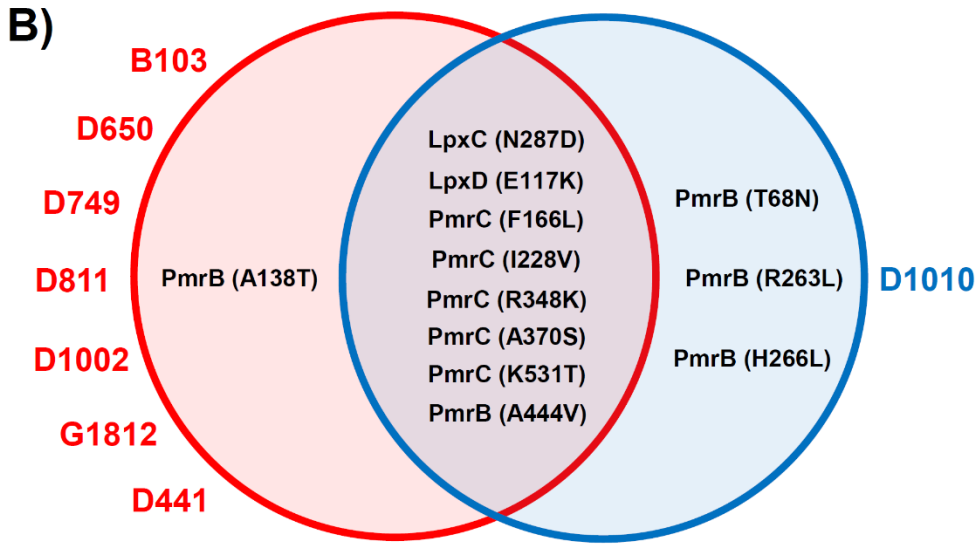

B)
B103
D650
D749
D811
D1002
G1812
D441
PmrB (A138T)
LpxC (N287D)
LpxD (E117K)
PmrC (F166L)
PmrC (I228V)
PmrC (R348K)
PmrC (A370S)
PmrC (K531T)
PmrB (A444V)
PmrB (T68N)
PmrB (R263L)
PmrB (H266L)
D1010

Fig. 1

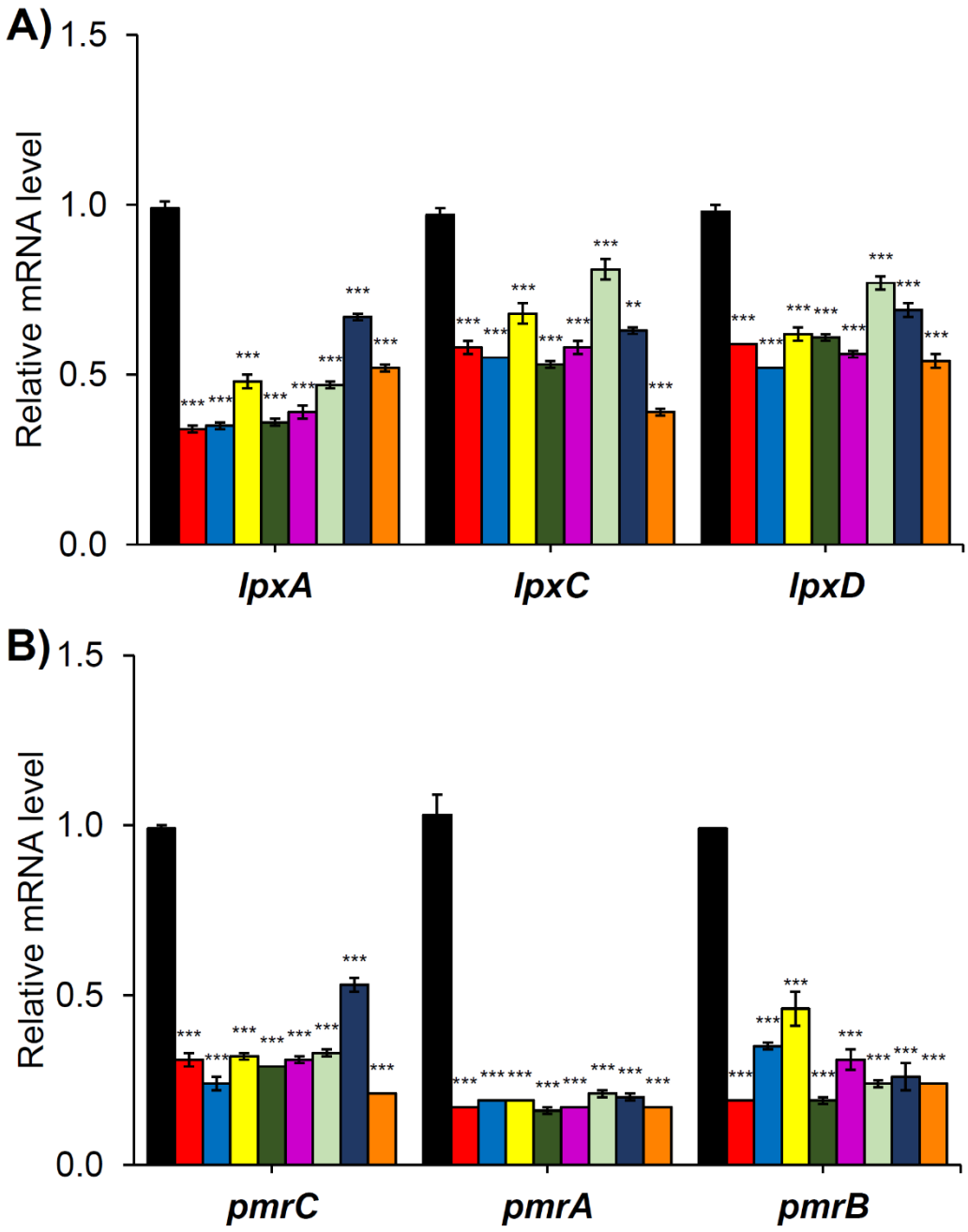

A)
Relative mRNA level
1.5
1.0
0.5
0.0
lpxA
lpxC
lpxD
B)
Relative mRNA level
1.5
1.0
0.5
0.0
pmrC
pmrA
pmrB

Fig. 2

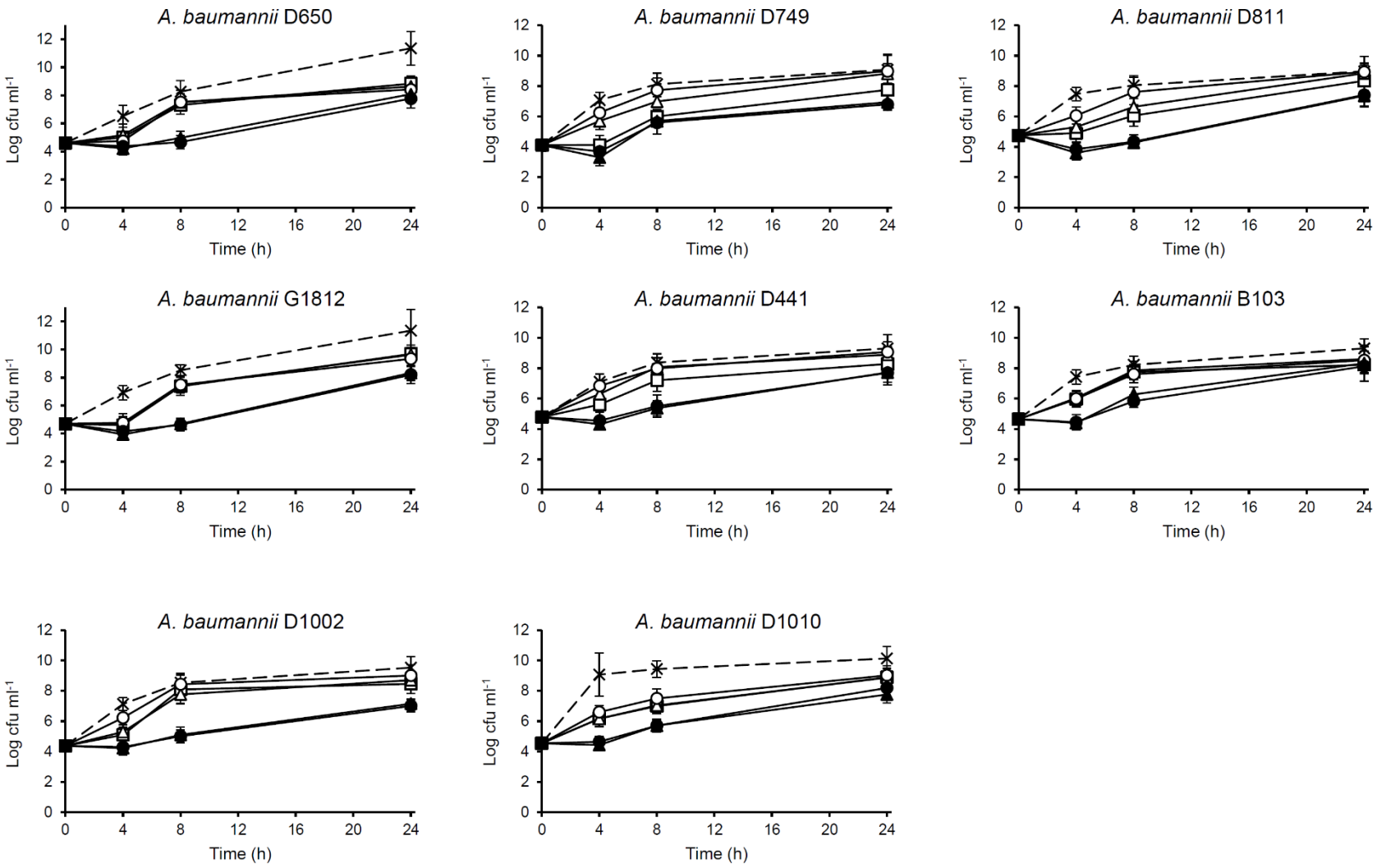

A. baumannii D650
A. baumannii D749
A. baumannii D811
A. baumannii G1812
A. baumannii D441
A. baumannii B103
A. baumannii D1002
A. baumannii D1010
Log cfu ml-1
Time (h)

Fig. 3

Table 1

Strain	ST*	Resistance profile†	Biological sample	MIC of CST (μg ml^{-1})	MIC of SeNPs (μg ml^{-1})	FICI of CST/SeNPs
ATCC 19606	52	WT	urine	0.5	256	0.502
Z6853	ND	AG, SXT, CB, FQ	bronchial aspirate	1	256	0.502
D988	ND	AG, SXT, CB, FQ	urine	0.5	256	0.502‡
D650	2	AG, SXT, CB, FQ, CST	aspiration catheter	16	128‡	**0.035**
D749	2	AG, SXT, CB, FQ, CST	wound	16	128‡	**0.035**‡
D811	2	AG, SXT, CB, FQ, CST	central venous catheter	32	256	**0.018**
G1812	2	AG, SXT, CB, FQ, CST	cervix swab	32	128	**0.012**
D441	2	SXT, CB, FQ, CST	aspiration catheter	64	128	**0.008**
B103	2	AG, SXT, CB, FQ, CST	wound	256	128‡	**0.006**
D1002	2	AG, SXT, CB, FQ, CST	urine	256	256	**0.004**‡
D1010	492	AG, SXT, CB, FQ, CST	aspiration catheter	256	256	**0.004**‡

*Sequence type according to MLST analysis (Pasteur). ND, not determined since the strain was not subjected to genomic analysis

†List of antibiotic classes against which the strain was resistant, as determined by VITEK® 2 system or broth microdilution method. WT, wild-type phenotype; AG, aminoglycosides; SXT, trimethoprim/sulfamethoxazole; CB, carbapenems; FQ, fluoroquinolones; CST, colistin

‡Formation of visible aggregates was observed, suggesting that isolate was starting to regrow