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Impairment of novel non-coding small RNA00203 inhibits biofilm formation and reduces biofilm-specific antibiotic resistance in *Acinetobacter baumannii*

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ABSTRACT

Small RNAs (sRNAs) are post-transcriptional regulators of many biological processes in bacteria, including biofilm formation and antibiotic resistance. The mechanisms by which sRNA regulates the biofilm-specific antibiotic resistance in *Acinetobacter baumannii* have not been reported to date. This study aimed to investigate the influence of sRNA00203 (53 nucleotides) on biofilm formation, antibiotic susceptibility, and expression of genes associated with biofilm formation and antibiotic resistance. The results showed that deletion of the sRNA00203-encoding gene decreased the biomass of biofilm by 85%. Deletion of the sRNA00203-encoding gene also reduced the minimum biofilm inhibitory concentrations for imipenem and ciprofloxacin 1024- and 128-fold, respectively. Knocking out of sRNA00203 significantly downregulated genes involved in biofilm matrix synthesis (*pgaB*), efflux pump production (novel00738), lipopolysaccharide biosynthesis (novel00626), preprotein translocase subunit (*secA*) and the CRP transcriptional regulator. Overall, the suppression of sRNA00203 in an *A. baumannii* ST1894 strain impaired biofilm formation and sensitized the biofilm cells to imipenem and ciprofloxacin. As sRNA00203 was found to be conserved in *A. baumannii*, a therapeutic strategy targeting sRNA00203 may be a potential solution for the treatment of biofilm-associated infections caused by *A. baumannii*. To the best of the authors' knowledge, this is the first study to show the impact of sRNA00203 on biofilm formation and biofilm-specific antibiotic resistance in *A. baumannii*.

1. Introduction

Acinetobacter baumannii has evolved from an opportunistic pathogen to a top-priority pathogen on the World Health Organization's priority list of antibiotic-resistant organisms for effective drug development [1]. Nowadays, *A. baumannii* is an important healthcare-associated pathogen. Rapid dissemination of this pathogen is attributed to its antibiotic resistance and biofilm formation properties [2,3]. The mortality rate of multi-drug-resistant *A. baumannii* ranges from 45.6% to 60.9%, and the mortality rate of extensively-drug-resistant strains can be as high as 84.3%, particularly in ventilator-associated pneumonia [4]. *A. baumannii* forms biofilm to endure adverse environments [2,5,6]. According to previous reports, 59.6–91.0% of *A. baumannii* strains were able to form biofilm [7,8]. Biofilm formation alters gene expression and results in the development of adaptive antibiotic resistance [5,9–11]. This is a cause of failure of antibiotic treatment of biofilm-associated infections [12,13]. The development of biofilm requires the concerted expression of multiple genes that are controlled by complex regulatory systems [14–16]. In *A. baumannii*, regulatory systems for biofilm formation include the two-component regulatory (*bfmR*) and quorum sensing systems (*abaI*) [17,18]. The *bfmRS* system regulates the expression of the *csuA/BABCDE* usher-chaperone assembly system, which is required for the attachment of cells to surfaces, and thereby facilitates biofilm formation on abiotic surfaces [18]. Similarly, the *abaI* and *abaR* genes regulate biofilm formation in response to population density [19]. The *abaI* gene encodes an autoinducer synthase that directs the production of autoinducer acyl-homoserine lactone, which binds to the cognate re-

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ceptor AbaR and activates expression of the *csu* operon and genes associated with biofilm production [19,20].

In addition to the two-component regulatory and quorum sensing systems, sRNAs play important roles in the post-transcriptional regulation of various biological processes including the formation of biofilms and the development of biofilm-associated antibiotic resistance [16,21–24]. sRNAs interact with the target mRNAs by forming base pairs at or near the ribosome binding sites of the target, which have either a positive or negative influence on various biological processes, including biofilm development, in the bacterial cells [5,23,25–31]. sRNA analysis of the RNA-sequencing data of the *A. baumannii* ATCC17978 and *A. baumannii* AB5075 strains revealed 31 and 78 putative sRNAs [32,33], respectively. Nonetheless, the roles of these sRNAs in the regulation of the post-transcriptional process of biofilm formation and biofilm-specific antibiotic resistance have not been reported to date. Álvarez-Fraga et al. reported that sRNA13573 identified from biofilm cells of *A. baumannii* ATCC 17978 was involved in biofilm formation and attachment of the bacterial cells to A549 alveolar epithelial cells [32]. However, the biological roles of sRNAs in the involvement of biofilm-specific antibiotic resistance in *A. baumannii* clinical strains have not been documented to date. The authors' previous transcriptome study of a clinical *A. baumannii* sequence type (ST)1894 strain revealed a panel of genes that were significantly upregulated in biofilm cells either untreated or treated with imipenem at the sub-minimum inhibitory concentration (MIC) compared with the planktonic cells [8,16]. The upregulated genes included sRNA00203 and genes involved in biofilm formation and antibiotic resistance, including *pgaB, secA*, novel00738, novel00626 and cAMP-activated global transcriptional regulator (CRP). sRNA00203 was most upregulated among the five differentially expressed sRNAs identified in the transcriptome study [16]. sRNA00203 consisted of 53 nucleotides, and showed complementarity with the mRNA sequences of genes involved in biofilm formation and antibiotic resistance [16]. Therefore, it was hypothesized that impairment of sRNA00203 in the *A. baumannii* ST1894 strain might affect the formation of biofilm and expression of antibiotic resistance genes specific to biofilm formation. This study aimed to investigate the impact of sRNA00203 on biofilm formation, antibiotic susceptibility, and expression of genes associated with biofilm formation and antibiotic resistance.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A clinical *A. baumannii* ST1894 strain was isolated from the sputum of a patient suffering from a lower respiratory tract infection. The *A. baumannii* ST1894 strain was susceptible to imipenem, ciprofloxacin and colistin when grown in the planktonic phase. However, this strain was identified as a hyper biofilm former and displayed a high degree of multi-drug tolerance to bactericidal antibiotics such as colistin, imipenem and ciprofloxacin when grown in the biofilm phase [8]. The *A. baumannii* ST1894 strain was cultured on Luria-Bertani (LB) agar at 37°C, and stored at -80°C in LB broth containing 20% glycerol.

2.2. Prediction of mRNA targets of sRNA 00203

sRNA00203 sequence (Table S1, see online supplementary material) was searched against the National Center for Biotechnology Information (NCBI) NR database using BLASTx. Novel transcript without annotation was considered as a potential sRNA candidate. BLASTn searches against the NCBI databases were used to evaluate whether the genes encoding sRNA00203 sequences were conserved across the *A. baumannii* strains. The IntaRNA algorithm was used to search for mRNA targets of sRNA00203 [34–37].

2.3. Construction of sRNA00203 knockout strain

sRNA00203 knockout strain was constructed using the double allelic exchange. Suicide vector pMo130-TelR containing kanamycin and tellurite resistance markers was used to delete the target gene according to the procedures described by Amin et al. [38] with some modifications. As shown in Figure 1, three overlapping DNA fragments harbouring the kanamycin resistance cassette [*aph(3')-I*, 795bp], the left flanking region (1000 bp upstream of sRNA00203) and the right flanking region (1001 bp downstream of sRNA00203) were amplified by polymerase chain reaction (PCR). The primers used to amplify the three fragments are shown in Table 1. The three overlapping PCR fragments were inserted into pMo130-TelR using a ClonExpress Ultra-One Step Cloning Kit (Vazyme Biotech Co. Ltd, Nanjing, China).

The mixture was mixed gently and incubated at 50°C for 50 min. Electrocompetent *A. baumannii* ST1894 cells were prepared using the protocol described by Richard Lab in OpenWetWare (https://openwetware.org/wiki/Richard_Lab:Preparing_electrocompetent_cells), and transformed with the recombinant plasmid by electroporation. Briefly, 10 µL of recombinant plasmid was mixed with 100 µL of electrocompetent cells on ice, and the contents were transferred to an electroporation cuvette (0.2-cm gap) on ice. The cuvette was placed in a Gene Pulser (Bio-Rad, Hercules, CA, USA), and the cells were subjected to a 2.5-kV electric pulse from a Gene Pulser (Bio-Rad) connected to a pulse controller (capacitance 25 µF; external resistance 200 Ω; time constant 4.5–5.0 ms). Immediately after electroporation, 100 µL of the electroporated content was transferred to 900 µL of LB broth and incubated at 37°C with agitation at 200 rpm for 1 h, followed by centrifugation at 5000 rpm for 5 min. After centrifugation, 900 µL of the supernatant was discarded, and the remaining 100 µL cell pellet was inoculated on to LB agar with 50 µg/mL kanamycin. The agar plate was incubated at 37°C for 72 h. Colonies that grew on LB agar with kanamycin were potential transformants, and were selected for confirmation of sRNA00203 gene deletion.

2.4. Confirmation of sRNA00203 knockout strain

Transformants were grown on LB agar containing 50 µg/mL kanamycin. A single colony was picked and subcultured in LB broth without kanamycin at 37°C for 24 h, and 10 passages were done to eliminate pMo130-TelR. A broth culture of the 10th passage was centrifuged at 5000 rpm for 5 min to obtain the cell pellet, from which DNA was extracted using a FavorPrep Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech. Corp, Ping Tung, Taiwan). The extracted genomic DNA was subjected to PCR and Sanger sequencing. To confirm the insertion of the kanamycin resistance gene (*aph(3')-I*) into the genome of *A. baumannii* ST1894, the junctions between the flanking regions and the *aph(3')-I* gene were amplified and sequenced. Primers LFKM203-F and LFKM-203R were used to amplify the left junction [the junction between the left flanking region of the sRNA00203 gene and the 5' end of *aph(3')-I*]. Similarly, primers KMRF203_2_F and KMRF203_2_R were used to amplify the right junction [the junction between the right flanking region of the sRNA00203 gene and the 3' end of *aph(3')-I*] (Table 1). A 461-bp purified PCR product of the right junction was sequenced using Sanger sequencing. Transformant with the *aph(3')-I* gene inserted between the left and right flanking regions of sRNA00203 was confirmed to be the knockout strain, and was stored in LB broth with 20% glycerol at -80°C until use.

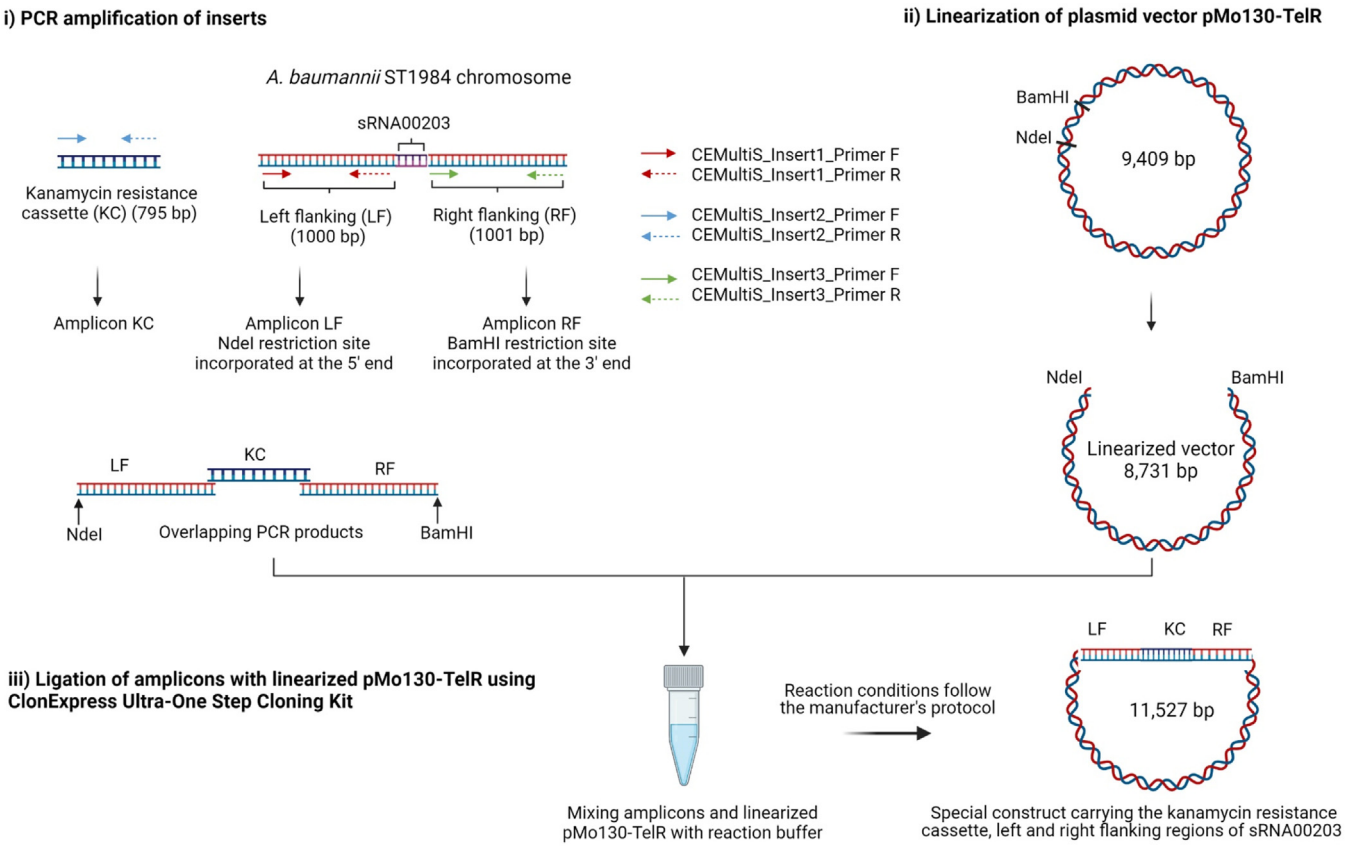

Figure 1. Construction of plasmid pMo130-TelR carrying the left flanking region (LF) (1000 bp upstream of sRNA00203), kanamycin resistance cassette (KC) (*aph(3′)-I*, 795 bp) and right flanking region (RF) (1001 bp downstream of sRNA00203). Ligation of the inserts with the linearized pMo130-TelR was performed using the ClonExpress Ultra-One Step Cloning Kit. Figure was created with BioRender.com. PCR, polymerase chain reaction.

Table 1
Primers used in the present study.

Primer name	Sequence (5′ 3′)	Use in the present study
CEMultiS_Insert1_Primer F (*Nde*I)	AGCGGGATCTGATGGCATATGCATTTGCCAAAGAGTATCAGTCTGC	Construction of ΔsRNA00203 strain: amplification of the 1000-bp left flanking region
CEMultiS_Insert1_Primer R	CTTGACGAGTTCTTCTGTTGAAATGAACGCTTAAGATTAGGA	
CEMultiS_Insert2_Primer F	CAACAGAAGAACTCGTCAAGAAGGCG	Construction of ΔsRNA00203 strain: amplification of the 795-bp kanamycin resistance cassette (*aph(3′)-I* gene)
CEMultiS_Insert2_Primer R	CGACGCGTGTGCATAAAAAATGATTGAACAAGATGGATTGCAC	
CEMultiS_Insert3_Primer F	TTTTTTATGCACACGCGTCGT	Construction of ΔsRNA00203 strain: amplification of the 1001-bp right flanking region
CEMultiS_Insert3_Primer R (*Bam*HI)	CGCATGCATCTAGAGGGATCCGCATCTTGCCATTCTTGGGTT	
KMRF203_F	GCTGCCTCGTCTTGGAGT	Confirmation of deletion in the ΔsRNA00203 strain
KMRF203_R	TAGCTGTCGTTGTGCCGTA	
LFKM203-F	CACACGACTTGCCATTGTTC	
LFKM203-R	CGGACCGCTATCAGGACATA	
sRNA203Fw-ndeI	CATATGCCGATATCAAGGAAGAGATCATAAGG	Complementation of the ΔsRNA00203 strain: cloning of sRNA00203-encoding gene into pWH1266 plasmid
sRNA203Rv-ndeI	CATATGCCTCTTGAAACTGTTCTTACTCAAGTTC	

Underlined sequences are the restriction enzyme recognition sites.
Restriction site BamHI was incorporated at the 5' terminus of the left flanking region (1000 bp upstream of sRNA00203). Another restriction site NdeI was incorporated at the 5' termini of the right flanking region (1001 bp downstream of sRNA00203). The same restriction enzymes were used to digest and linearize the plasmid vector (pMo130-TelR). The three purified polymerase chain reaction products and the linearized vector were mixed with the 2X ClonExpress Mix in an appropriate ratio. Optimal volumes of the inserts and linearized vector were determined using the formula given in the ClonExpress Ultra One Step Cloning Kit manual (http://www.vazyme.com).

2.5. Complementation of stable mutant with sRNA00203

The gene encoding sRNA00203 was amplified from the *A. baumannii* ST1894 genomic DNA. NdeI restriction sites were incorporated into the 5' termini of the primers sRNA203Fw-ndeI and sRNA203Rv-ndeI (Table 1). The PCR product was purified using a Monarch PCR and DNA Cleanup Kit (New England BioLabs, Hitchin). A 2-µg aliquot of purified PCR product was digested with NdeI following the manufacturer's protocol (New England BioLabs). The NdeI-digested PCR products were then purified using the Monarch PCR and DNA Cleanup Kit (New England BioLabs).

The pWH1266-TelR plasmid (a generous gift from Margarita Poza Dominguez, Complexo Hospitalario Universitario, Spain), which contains tellurite and tetracycline markers, was used as

a shuttle vector. The *Eschericha coli* host carrying pWH1266-TelR was grown on the LB agar plate containing 50 µg/mL potassium tellurite. A single *E. coli* colony was subcultured in LB broth containing potassium tellurite at 37°C for 24 h. The plasmid was then extracted from the broth culture using a QIAfilter Plasmid Midi Kit (Qiagen, Hilden, Germany), purified, and linearized by digestion with NdeI (New England BioLabs). After digestion, 3 µg of the linearized pWH1266-TelR was treated with quick calf-intestinal alkaline phosphatase (CIP) (New England BioLabs) to prevent plasmid self-ligation and enable the successful ligation of the target fragment with the sRNA00203-encoding gene. CIP-treated and linearized pWH1266-TelR was purified using a Monarch PCR and DNA Cleanup Kit (New England BioLabs). The NdeI-digested PCR product of the sRNA00203-encoding gene was cloned into linearized pWH1266-TelR using T4 DNA ligase (New England BioLabs) in accordance with the manufacturer's protocol. Ten microlitres of the ligation reaction was mixed with 100 µL of competent cells prepared from the sRNA00203-knockout *A. baumannii* ST1894, using the protocol described by Richard Lab in Open Wetware (https://openwetware.org/wiki/Richard_Lab:Preparing_electrocompetent_cells). The pWH1266-TelR carrying sRNA00203 was transformed into the sRNA00203-knockout *A. baumannii* ST1894 strain using electroporation as described in Section 2.3. Transformants were selected by growing the bacterial cells on LB agar plates with 25 µg/mL tetracycline and 50 µg/mL kanamycin for 48 h at 37°C. PCR was performed to confirm the success of sRNA00203 complementation using a pair of primers sRNA203Fw-ndeI and sRNA203RV-ndeI to detect the gene encoding sRNA00203. Reverse transcription PCR was performed to verify the expression of sRNA00203 of the complemented strain according to the procedures described in Sections 2.8 and 2.9.

2.6. Biofilm formation assay

The biofilm formation capabilities of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains of *A. baumannii* ST1894 were tested using the Calgary Biofilm Device (Innovotech, Edmonton, Canada) following procedures described by Ceri et al. [39]. In brief, three to five colonies were selected from an overnight culture on the LB agar plate. The colonies were inoculated into 10 mL of Maximum Recovery Diluent with turbidity adjusted to the McFarland standard 0.5. In a 96-well microtitre plate, 150 µL of bacterial suspension of the wild-type, sRNA00203-mutant or sRNA00203-complemented strains of *A. baumannii* ST1894 was transferred into a well. Next, a plastic cap with 96 pegs was placed on top of the microtitre plate. The microtitre plate was incubated at 37°C with agitation at 110 rpm for 48 h. Planktonic cells were aspirated from the wells after incubation and discarded. The wells were washed three times with Maximum Recovery Diluent. The plates and lids with pegs were positioned upside down and dried at room temperature for 2 h. Biofilm masses formed on a 96-well microlitre plate were quantified using the crystal violet staining method, as described previously [8,40]. The mean optical densities (OD) of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were determined, and the biofilm formation capability was defined following the guidelines mentioned by Ceri et al. [40].

2.7. Antibiotic susceptibility test for biofilms

The antibiotic susceptibility test for biofilm cells was evaluated following procedures described by Moskowit et al. [41]. The minimum biofilm inhibitory concentrations (MBIC) for imipenem and colistin in the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were determined using broth microdilution techniques, as described by Shenkutie et al. [8]. In addition, the minimum biofilm eradication concentrations (MBEC) for imipenem, ciprofloxacin and colistin in the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were measured using the Calgary Biofilm Device in accordance with the procedures described by Moskowit et al. [8].

2.8. RNA extraction

Bacterial cells harvested from the planktonic and biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were processed for total RNA isolation using a PureLink RNA Mini Kit (Ambion, Life Science Technologies, Carlsbad, CA, USA). The isolated RNA was treated with TURBO DNase (Life Science Technologies) to eliminate contaminating genomic DNA.

2.9. Quantification of expression levels of sRNA00203 and genes related to biofilm formation and antibiotic resistance by reverse transcription PCR

RNA samples were extracted from the planktonic and biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains and reverse transcribed into cDNA using a LunaScript RT Supermix Kit (New England BioLabs), followed by TaqMan qPCR assay using a Luna Universal Probe qPCR Master Mix (New England BioLabs). The primers and probe sequences used to confirm the expression genes related to biofilm formation and antibiotic resistance are shown in a study conducted by Shenkutie et al. [16]. *gyrB* gene was used as an internal control to normalize the expression of target genes. Expression levels of the target genes in the wild-type, sRNA00203-mutant and sRNA00203-complemented biofilm cells relative to the wild-type strain planktonic cells were calculated using the $2^{-\Delta\Delta Ct}$ method [42].

2.10. Statistical analyses

Gene expression data were analysed using GraphPad Prism Version 9.5.1 (GraphPad Software Inc, San Diego, CA, USA) and are presented as mean ± standard deviation. Shapiro–Wilk test was used to test the normality of data distribution. The mean OD at 570 nm and fold changes in the expression levels of the target genes were compared between the wild-type, sRNA00203-mutant and sRNA00203-complemented biofilm cells using one-way analysis of variance. If the difference was significant ($P<0.05$), a post-hoc test was conducted between datasets to identify pairs with significant differences. Tukey's post-hoc test was used to analyse the data and compare differences between each paired category. $P< 0.05$ was considered to indicate significance.

3. Results

3.1. Characterization of sRNA00203

The sRNA00203 gene sequence was searched against the NCBI NR database using BLASTx, which revealed that the gene sequence was devoid of any recognizable peptide-coding sequence. Besides, the sequence encoding sRNA00203 was located in the intergenic region. This showed that sRNA00203 belonged to non-coding sRNA. The whole sequence of sRNA00203 was found conserved between 7557 *A. baumannii strains* deposited in the NCBI GenBank database. Complementary base pairing between sRNA and mRNA for genes involved in biofilm formation and biofilm-specific antibiotic resistance in *A. baumannii* ST1894 was predicted using the IntaRNA algorithm. The base pairing positions of sRNA00203 and the target mRNA are shown in Table 2.

Table 2
Complementary base pairing between sRNA and mRNA for genes involved in biofilm formation and biofilm-specific antibiotic resistance predicted using the intaRNA algorithm.

mRNA_ID	KEGG_ID	Strand	Start	End	sRNA00203 position	mRNA position	Energy (kcal/mol)	Pathways
E5A72_RS12100 (*pgaB*)	acb: 1S_0938	+	2520896	2522890	31 – 41	1584 – 1594	-9.51842	Biofilm matrix
Novel00738	acb: 1S_2736	+	617454	623989	6 – 27	461 – 492	-15.12	β-lactam resistance Cationic antimicrobial peptide resistance
Novel00626 (*lpxA*)	acb: 1S_1965	-	3676375	3677683	25 – 34	265 – 274	7.86	Cationic antimicrobial peptide resistance Lipopolysaccharide biosynthesis
E5A72_RS03500 (*secA*)	acb: 1S_2862	-	756066	758789	7 – 50	2653 – 2695	-8.60926	Quorum sensing Protein export Bacterial secretion system
E5A72_RS13515 (cAMP-activated global transcriptional regulator CRP)	acb: 1S_1182	-	2818333	2819040	8 – 48	204 – 244	-11.0242	Two-component system Quorum sensing

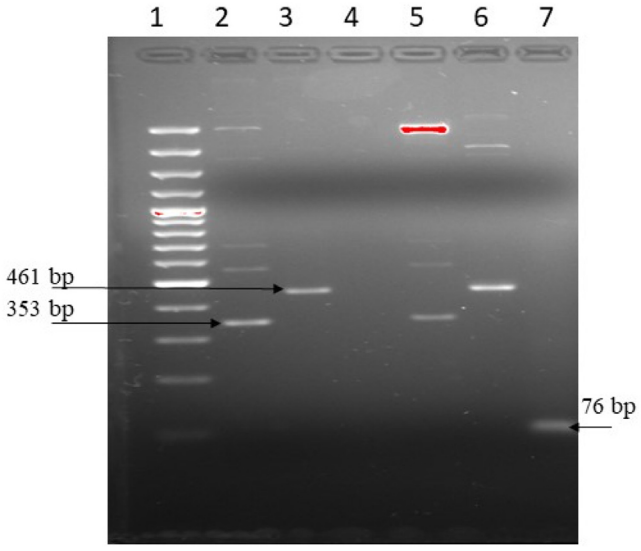

Figure 2. Polymerase chain reaction (PCR) amplification of the junctions between the *aph(3')-I* gene (kanamycin resistance cassette) and the left and right flanking regions of the sRNA00203 gene. Lane 1: 100bp Plus DNA ladder. Lanes 2–4: PCR results of the sRNA00203-mutant strain; Lane 2: left flanking region and kanamycin resistance cassette (353 bp); Lane 3: right flanking region and kanamycin resistance cassette (461 bp); Lane 4: sRNA00203. Lanes 5–7: PCR results of the sRNA00203-complemented strains; Lane 5: left flanking region and kanamycin cassette (353 bp); Lane 6: right flanking region and kanamycin cassette (461 bp); Lane 7: sRNA00203 (76 bp).

3.2. Confirmation of sRNA00203 gene knockout in *A. baumannii* ST1894

Replacement of the gene encoding sRNA00203 by the *aph(3')-I* gene (kanamycin resistance cassette) was performed through allelic exchange using the pMo130-TelR plasmid. Successful knockout of the sRNA00203 gene in *A. baumannii* ST1894 was confirmed by amplifying the junctions between: (i) the left flanking region of sRNA00203 and the 5' terminus of the *aph(3')-I* gene (amplicon size 353 bp); and (ii) the right flanking region of sRNA00203 and the 3' terminus of the *aph(3')-I* gene (amplicon size 461 bp) (Figure 2). The sequencing result of the 461-bp amplicon confirmed the presence of the 3' terminus of *aph(3')-I* at the right flanking region of the sRNA00203-encoding gene (Figure 3).

Furthermore, when compared with the wild-type planktonic cells, the expression fold change of the sRNA00203 gene in the biofilm cells of the knockout strains was 0.49, which was significantly lower than that of the wild-type strain (expression fold change 5.49, P=0.005) and the complemented strain (expression fold change 4.83, P<0.001) (Figure 4).

3.3. Effect of sRNA00203 inactivation on biofilm formation

The biofilm formation capabilities of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains were compared, and the results are shown in Figure 5. Deletion of the sRNA00203-encoding gene decreased the biofilm mass by 85% compared with that of the wild-type strain (P<0.0001). The complementation of sRNA00203 restored the biofilm formation capability to a level comparable with that of the wild-type strain (Figure 5). These findings suggested that sRNA00203 participates in the regulation of biofilm formation in *A. baumannii* ST1894.

3.4. Influence of sRNA00203 on the antibiotic susceptibility of biofilms

The MICs and MBICs for imipenem, ciprofloxacin and colistin were compared between the wild-type, sRNA00203-mutant and sRNA00203-complemented strains. When grown in the planktonic phase, all three strains were susceptible to the three antibiotics tested (Table 3). For each antibiotic, the MICs were the same between different strains, suggesting that deletion of the sRNA00203-encoding gene did not affect the susceptibility of planktonic cells to the three antibiotics. When grown in the biofilm phase, the MBIC of imipenem for the sRNA00203-mutant strain decreased 1024-fold and 512-fold compared with the wild-type and sRNA00203-complemented strains, respectively (Table 3). Similarly, the MBIC of ciprofloxacin for the sRNA00203-mutant strain decreased 128-fold and 64-fold compared with the wild-type and sRNA00203-complemented strains, respectively (Table 3). When the gene encoding sRNA00203 was deleted, the *A. baumannii* ST1894 biofilm became susceptible to imipenem and ciprofloxacin. Deleting the gene also reduced the MBIC of colistin by half compared with the wild-type and sRNA00203-complemented strains (Table 3). However, the biofilm cells of the sRNA00203-mutant strain remained resistant to colistin.

3.5. Effect of sRNA00203 deletion on the antibiotic tolerance of biofilms

The MBEC assay was performed to evaluate the antibiotic concentrations required to eradicate biofilm cells of wild-type, sRNA00203-mutant and sRNA00203-complemented *A. baumannii* ST1894 (Table 4). Similar to the MICs, the MBCs of each antibiotic were the same for the different strains, showing that dele-

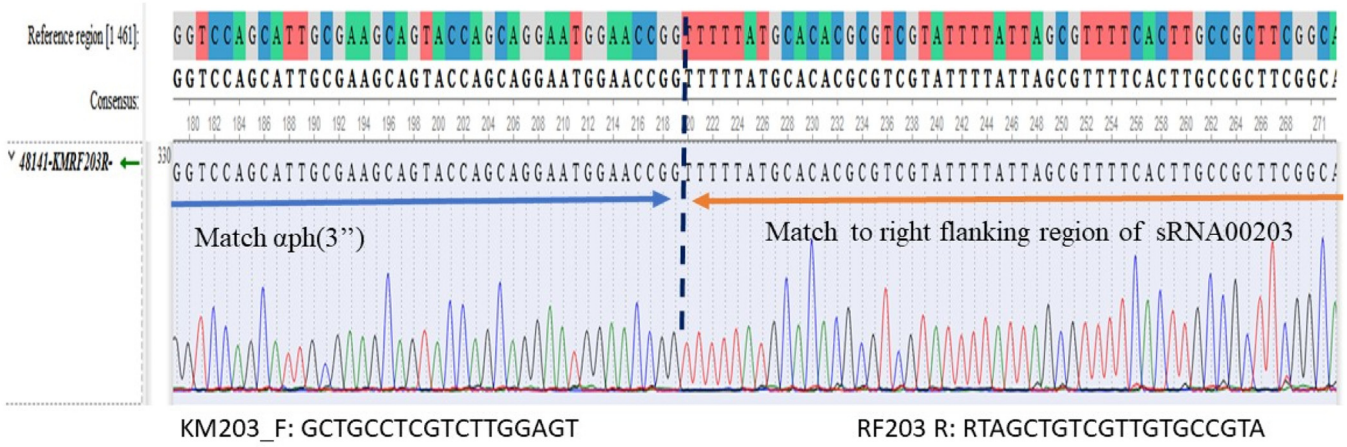

Figure 3. Verification of sRNA00203 knockout in *Acinetobacter baumannii* ST1894. Sanger sequencing showed that the 3' terminus of *aph(3')-I* gene (kanamycin resistance cassette) was fused with the right flanking region of the sRNA00203 gene, which indicated the replacement of sRNA00203 with the *aph(3')-I* gene.

Table 3
Impact of sRNA00203 deletion on the antibiotic susceptibility of *Acinetobacter baumannii* ST1894 biofilms.

A. baumannii ST1894 strains	Imipenem		Ciprofloxacin		Colistin	
	MIC for planktonic cells (μg/mL)	MBIC for biofilm cells (μg/mL)	MIC for planktonic cells (μg/mL)	MBIC for biofilm cells (μg/mL)	MIC for planktonic cells (μg/mL)	MBIC for biofilm cells (μg/mL)
Wild-type	0.125	256	1	64	0.5	32
sRNA00203-mutant	0.125	0.25	1	0.5	0.5	16
sRNA00203-complemented	0.125	128	1	32	0.5	32

MIC, minimum inhibitory concentration; MBIC, minimum biofilm inhibitory concentration.

Table 4
Impact of sRNA00203 deletion on biofilm eradication by antibiotics.

A. baumannii ST1894 strains	Imipenem		Ciprofloxacin		Colistin	
	MBC (μg/mL)	MBEC (μg/mL)	MBC (μg/mL)	MBEC (μg/mL)	MBC (μg/mL)	MBEC (μg/mL)
Wild-type	4	>1,024	8	>1,024	4	256
sRNA00203-mutant	4	8	8	1,024	4	256
sRNA00203-complemented	4	1024	8	512	4	128

MBC, minimum bactericidal concentration (performed in planktonic cells); MBEC, minimum biofilm eradication concentration.

tion of the sRNA00203-encoding gene did not affect the minimum antibiotic dosage for eradication of the planktonic cells. The MBEC of imipenem for the sRNA00203-mutant was 8 μg/mL, which was approximately a 128-fold reduction compared with the wild-type and sRNA00203-complemented strains. The MBECs of ciprofloxacin and colistin for the sRNA00203-mutant were 1024 μg/mL and 256 μg/mL, respectively (Table 4), which was about the same as with the wild-type strain but a two-fold increase compared with the complemented strain. Overall, deletion of the sRNA00203-encoding gene reduced the MBEC of imipenem substantially, but not ciprofloxacin or colistin.

3.6. Impact of sRNA00203 on the expression of genes associated with biofilm formation and antibiotic resistance

The influence of sRNA00203 on the expression levels of a panel of five genes associated with biofilm formation and antibiotic resistance was assessed (Figure 6). The expression level of *pgaB* was 14.5 times higher in biofilm cells than in planktonic cells of the wild-type strain. When the sRNA00203-encoding gene was deleted, the expression of *pgaB* in biofilm cells increased only 1.42-fold compared with that in planktonic cells (Figure 6). In the sRNA00203-complemented biofilms, *pgaB* expression was 13.7 times higher than in the planktonic cells of the wild-type strain. These results indicate that deletion of sRNA00203 significantly reduced the expression of *pgaB,* which, in turn, might reduce the synthesis of biofilm matrix ($P<0.0001$).

The novel00738 gene, which is associated with the resistance–nodulation–cell division (RND) family drug transporters, was expressed differentially in *A. baumannii* ST1894 biofilm cells compared with planktonic cells [16]. The expression levels of novel00738 in biofilm cells were 29.2-fold, 3.83-fold and 33.3-fold higher than those in the planktonic cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains, respectively. These findings indicate that deletion of the sRNA00203 gene significantly reduced the expression of novel00738 in the mutant strain compared with the wild-type ($P<0.0001$) and sRNA00203-complemented strains ($P<0.0001$) (Figure 6).

The novel00626 gene is primarily involved in metabolic pathways such as lipopolysaccharide biosynthesis and cationic antimicrobial peptide resistance. When sRNA00203 was knocked out, the expression of novel00626 was 2.2-fold lower in biofilm cells than in planktonic cells (Figure 6). The expression of novel00626 was 30.8-fold and 25.8-fold higher in biofilm cells than in planktonic cells of the wild-type and sRNA00203-complemented strains, respectively.

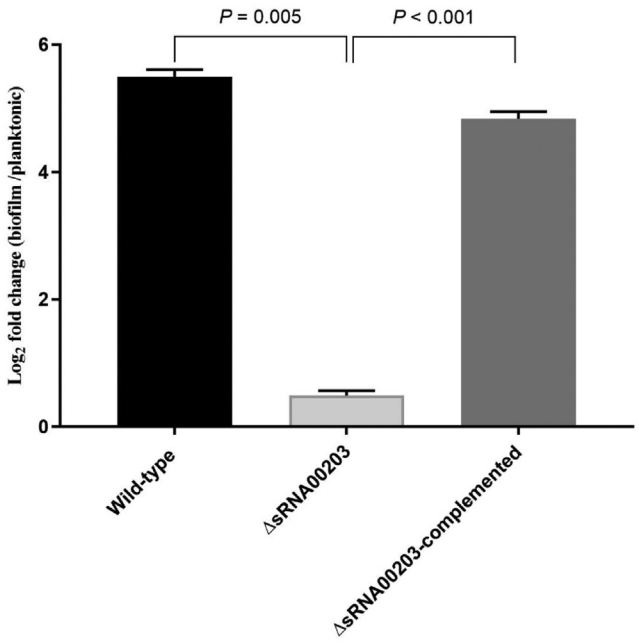

Figure 4. Expression levels of sRNA00203 in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented *Acinetobacter baumannii* ST1894 strains. The mean $\log_2$ fold changes in the three biofilm groups relative to planktonic cells of the wild-type strain are shown. Three independent biological replicates were evaluated for each group. The error bars represent standard deviations between different replicates. Analysis of variance and a post-hoc test was performed to evaluate the statistical differences between the three groups. $P<0.05$ was considered to indicate significance.

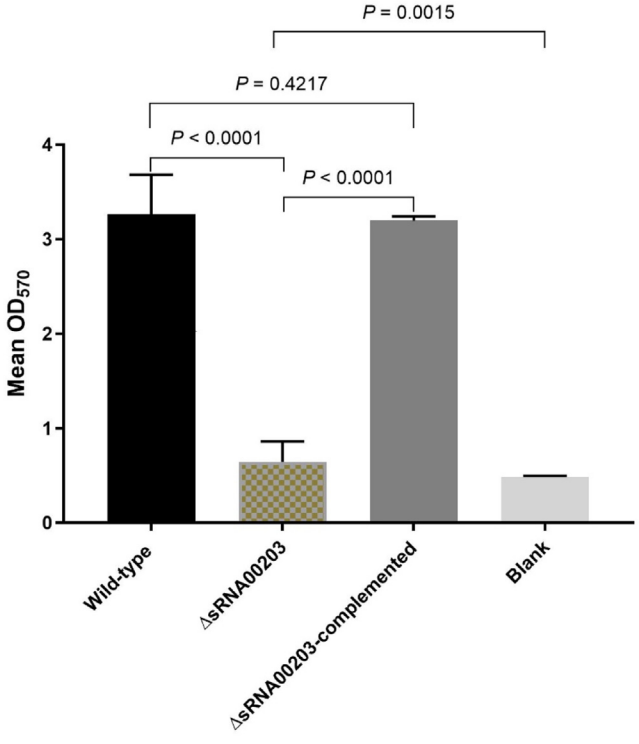

Figure 5. Effect of sRNA00203 inactivation on the biofilm formation capabilities of the wild-type, sRNA00203-mutant and sRNA00203-complemented *Acinetobacter baumannii* ST1894 strains. OD, optical density.

Expression of the gene encoding the preprotein translocase subunit SecA was 63.5-fold, 2.6-fold and 58.2-fold higher in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains, respectively, compared with the corresponding planktonic cells. In other words, the expression of *secA* decreased by 94% when sRNA00203 was deleted from the wild-type *A. baumannii* strain ($P<0.0001$; Figure 6).

Expression levels of the gene encoding the CRP transcriptional regulator were 34.7-fold, 2.9-fold and 32.2-fold higher in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented strains, respectively, compared with the corresponding planktonic cells. The sRNA00203 knockout strain, therefore, showed a substantial decrease in the expression of this gene compared with the wild-type ($P<0.0001$) and sRNA00203-complemented strains ($P<0.0001$; Figure 6).

4. Discussion

This study reports the influence of sRNA00203 identified from *A. baumannii* ST1894 on biofilm formation, antibiotic susceptibility of biofilm, and expression levels of genes associated with biofilm and antibiotic resistance. Deletion of sRNA00203 was found to reduce the biofilm mass by 85% compared with the wild-type *A. baumannii* ST1894 strain. Complementation of the mutant with the sRNA00203 gene restored its biofilm formation capacity to a level similar to that of the wild-type strain. Álvarez-Fraga et al. (2017) reported the role of sRNA13573 in regulating attachment to and biofilm formation on human A549 cells [32]. The present study demonstrated the involvement of sRNA00203 in biofilm-specific antibiotic resistance in addition to biofilm formation.

The present results showed that the MBICs of ciprofloxacin and imipenem for the sRNA00203-mutant were 128-fold and 1024-fold, respectively, lower than those in the wild-type *A. baumannii* ST1894 strain. sRNA00203 complementation restored the MBICs for ciprofloxacin and imipenem to values similar to those in the wild-type strain. These results suggest that deletion of the gene encoding sRNA00203 made the biofilm cells more sensitive to ciprofloxacin and imipenem, and may enhance the efficacy of both antibiotics in the treatment of biofilm-specific *A. baumannii* infections. These findings also indicated that sRNA00203 was involved in regulating the expression of genes responsible for ciprofloxacin and imipenem resistance in *A. baumannii* biofilm. On the other hand, deletion of the gene encoding sRNA00203 had no significant influence on the MBIC of colistin. It is possible that sRNA00203 is not involved in the regulation of genes associated with colistin resistance.

This study further evaluated the role of sRNA00203 in the viability of biofilm cells after treatment with ciprofloxacin, imipenem and colistin. The MBEC for imipenem in the sRNA00203-mutant strain was approximately 128-fold lower than that in the wild-type strain, and the MBEC was restored in the sRNA00203-complemented strain. However, the deletion of sRNA00203 did not significantly reduce the MBECs for colistin and ciprofloxacin. These findings suggest that sRNA00203 is involved in regulating the expression of genes involved in biofilm formation, which, in turn, affects antibiotic tolerance when biofilms are exposed to imipenem. Thus, when sRNA00203 is blocked, imipenem can effectively eradicate acinetobacter biofilm cells. Intriguingly, the sequence of sRNA00203 is conserved across all 7557 *A. baumannii* sequences deposited in the NCBI database, suggesting that sRNA00203 could be used as a therapeutic target for biofilm-specific infections caused by *A. baumannii*.

Based on the computational prediction using IntaRNA, this study found that sRNA00203 had binding sites com-

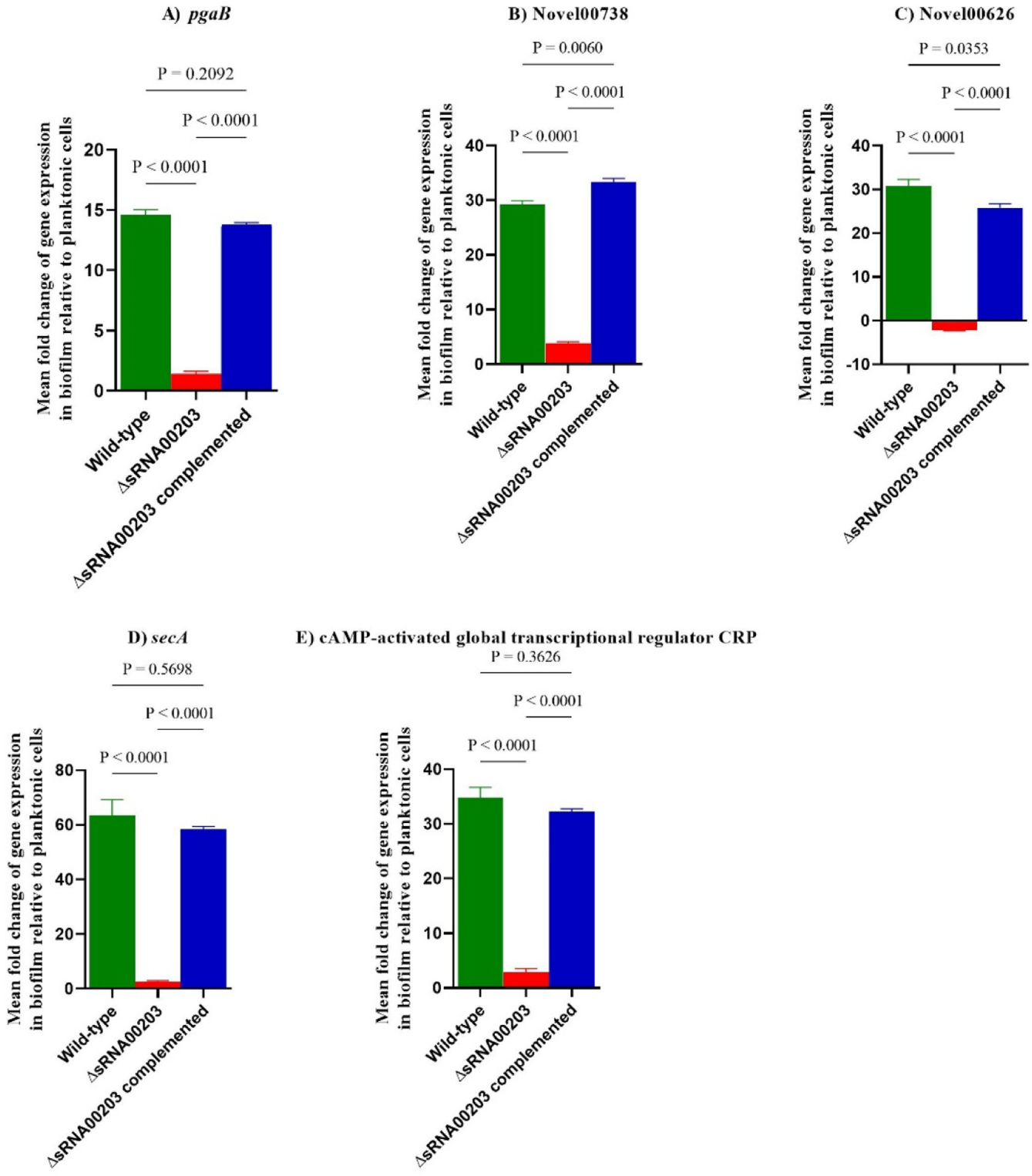

Figure 6. Expression levels of biofilm-specific genes (*pgaB,* novel00738, novel00626, *secA, CRP)* in biofilm cells of the wild-type, sRNA00203-mutant and sRNA00203-complemented *Acinetobacter baumannii* ST1894 strains. The mean fold changes in the three biofilm groups relative to planktonic cells of the wild-type strain are shown. Three independent biological replicates were analysed for each group. The bars represent standard error of mean between different replicates. Analysis of variance and post-hoc tests were performed to assess the statistical differences between the three groups. $P<0.05$ was considered to indicate significance.

plementary to the mRNAs of the genes encoding for *pgaB*, novel00738, novel00626, *secA* and CRP transcriptional regulator. This suggests that sRNA00203 may have a regulatory role for these genes. The gene knockout experiment showed that the deletion of sRNA00203 decreased the expression of *pgaB*, novel00738, novel00626, *secA* and CRP transcriptional regulators in biofilm cells significantly. The complementation of sRNA00203 restored the expression levels of the five genes, showing the positive regulatory role of sRNA00203 for these genes.

pgaB is involved in the biofilm matrix synthesis [43,44]. sRNA00203 may regulate biofilm formation by interacting with the mRNA of *pgaB*, which, in turn, reduced the amount of biofilm formed. The novel00738 gene encodes a drug efflux pump, the RND family drug transporter, which potentially confers resistance to ß-lactams. In the authors' previous transcriptome study, the expression level of novel00738 was much higher in imipenem-treated biofilm cells compared with untreated biofilm cells [16]. Deletion of sRNA00203 reduced the expression of novel00738, which might increase the susceptibility of biofilm cells to the antibiotic, as demonstrated by the decreased MBICs in the sRNA00203-mutant strain. This finding has high clinical significance, particularly for the treatment of biofilm-associated infections [44,45]. However, further investigations are needed to understand the exact mechanisms involved in the regulation of antibiotic susceptibility in biofilm cells by sRNA00203 and novel00738.

The novel00626 gene (*lpxA*) encodes UDP-N acetyl glucosamine O-acyltransferase, which is involved in the lipopolysaccharide biosynthesis pathway. Deletion of sRNA00203 reduced the biosynthesis of lipopolysaccharide, which, in turn, reduced biofilm formation. Previous studies have shown that expression of *lpxA* facilitates pathogens to survive the desiccation [46], and deletion of sRNA00203, which reduces the expression of novel00626, may also prevent the pathogen from surviving extended periods of desiccation.

Both genes encoding the SecA and CRP transcriptional regulators regulate the production of molecules involved in the quorum sensing system [47–49]. Deletion of sRNA00203 may reduce the quorum sensing molecules and therefore indirectly decrease biofilm formation and biofilm-specific antibiotic tolerance. Further investigations are required to understand how sRNA00203 interacts with these two genes.

To the best of the authors' knowledge, this is the first study to show a link between sRNA00203 with biofilm formation and biofilm-specific antibiotic resistance in *A. baumannii*. It showed that deletion of sRNA00203-encoding genes significantly decreased the biofilm formation capabilities of *A. baumannii*, and increased the susceptibility of biofilm cells to ciprofloxacin and imipenem. These properties could be a consequence of the decreased expression of genes involved in biofilm formation and biofilm-specific antibiotic resistance. As sRNA00203 was found to be conserved in *A. baumannii*, a therapeutic strategy targeting sRNA00203 may be a potential solution for the treatment of biofilm-associated infections caused by *A. baumannii*.

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Ethical approval: Not required.

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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.2023.106889.

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