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Global regulator SoxR is a negative regulator of efflux pump gene expression and affects antibiotic resistance and fitness in *Acinetobacter baumannii*

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Abstract

SoxR is a global regulator contributing to multidrug resistance in *Enterobacteriaceae*. However, the contribution of SoxR to antibiotic resistance and fitness in *Acinetobacter baumannii* has not yet been studied. Comparisons of molecular characteristics were performed between 32 multidrug-resistant *A. baumannii* isolates and 11 susceptible isolates. A *soxR* overexpression mutant was constructed, and its resistance phenotype was analyzed. The impact of SoxR on efflux pump gene expression was measured at the transcription level. The effect of SoxR on the growth and fitness of *A. baumannii* was analyzed using a growth rate assay and an *in vitro* competition assay. The frequency of the Gly39Ser mutation in *soxR* was higher in multidrug-resistant *A. baumannii*, whereas the *soxS* gene was absent in all strains analyzed. *SoxR* overexpression led to increased susceptibility to chloramphenicol (4-fold), tetracycline (2-fold), tigecycline (2-fold), ciprofloxacin (2-fold), amikacin (2-fold), and trimethoprim (2-fold), but it did not influence imipenem susceptibility. Decreased expression of *abeS* (3.8-fold), *abeM* (1.3-fold), *adeJ* (2.4-fold), and *adeG* (2.5-fold) were correlated with *soxR* overexpression ($P < .05$). However, the expression levels of *adeB* and *craA* showed no obvious difference in the *soxR*-overexpression mutant. Competitive growth test results showed that *soxR* overexpression led to a lower growth rate, which was associated with a significant fitness cost *in vitro*. These results reveal that the global regulator SoxR is a negative regulator of efflux pump gene expression, and contributes to antibiotic resistance and fitness in *A. baumannii*.

Abbreviations: CFU = colony-forming units, CI = competitive index, CRAB = carbapenem-resistant *A. baumannii*, CSAB = carbapenem-susceptible *A. baumannii*, LB = Luria–Bertani, MATE = multidrug and toxic compound extrusion, PCR = polymerase chain reaction, RND = resistance-nodulation-division, RT-PCR = reverse transcription PCR, SMR = small multidrug resistance.

Keywords: *Acinetobacter baumannii*, antibiotic resistance, efflux pump, global regulator, SoxR

1. Introduction

Acinetobacter baumannii is an important opportunistic pathogen that commonly causes nosocomial infections, such as ventilator-associated pneumonia and skin, soft tissue, wound, and bloodstream infections.[1] Multidrug-resistant *A. baumannii* strains have been increasingly reported worldwide, which raises serious concerns about the limited antimicrobial treatment options available.[2] SoxR is a global repressor protein involved in bacterial antibiotic resistance, and it contributes to multidrug-resistant phenotypes in *Enterobacteriaceae*.[3,4] SoxR protein induces the overexpression of efflux systems by activating the expression of the *soxS* gene in *Enterobacteriaceae*, or direct regulation in *Pseudomonas aeruginosa*.[5] However, the contribution of SoxR to antibiotic resistance and fitness in *A. baumannii* has not yet been studied. Here we focused on the role of the global regulator SoxR in *A. baumannii*. Comparisons of molecular characteristics were performed between carbapenem-resistant *A. baumannii* (CRAB) and carbapenem-susceptible *A. baumannii* (CSAB) isolates. A *soxR*-overexpression mutant was constructed, and the resistance phenotype and expression of multiple efflux pump genes were measured. We also analyzed the contribution of *soxR* overexpression to the growth and fitness of *A. baumannii*.

2. Materials and methods

2.1. Bacterial strain isolation and susceptibility testing

Forty-three *A. baumannii* clinical isolates were collected from the Chinese Antimicrobial Resistance Surveillance of Nosocomial Infections in 2011, including 11 carbapenem-susceptible isolates and 32 carbapenem-resistant isolates. *A. baumannii* was primarily identified using the VITEK2 system (bioMérieux, Marcy l' toile, France), and confirmed by the presence of a $bla_{\text{OXA51-like}}$ gene. Susceptibility testing of antimicrobials was performed by the agar dilution method as described previously.[6] The study was approved by the Institutional Review Board of Peking University People's Hospital.

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2.2. Polymerase chain reaction (PCR) and nucleotide sequencing

PCR amplification was performed using a 7300 thermocycler (Applied Biosystems, Foster City, CA). Primers were designed to amplify the full-length *soxR* and *soxS* sequences. Sequencing of the products was performed by an ABI 3730 DNA analyzer (Applied Biosystems) and analyzed using CLC sequence viewer software (CLC bio, Aarhus, Denmark).

2.3. Gene expression analysis using real-time reverse transcription PCR (RT-PCR)

Primers targeting *adeB*, *adeJ*, *adeG*, *craA*, *abeM*, and the housekeeping gene *rpoB* were used as described previously.[7–9] Primers were designed in this study for *soxR* (5′-ATGGATATTGGTGAAGTCG-3′ and 5′-TTAAAGTTTTGTTGGCTGAT-3′) and *abeS* (5′-TTTGGTCAGGCGCAGGTATT-3′ and 5′-ACCAATGCAGGCAGCTAAGT-3′). Bacteria were grown aerobically in Luria–Bertani (LB) broth until mid-log phase. DNase-treated RNA templates were prepared using the RNeasy Kit (Qiagen, Hilden, Germany). cDNA was generated from total RNA using random primer hexamers. RT-PCRs were performed using a 7300 thermocycler (Applied Biosystems) with a SYBR green PCR master mix (TaKaRa, Tokyo, Japan). The PCR program consisted of 5 seconds at 95°C, followed by 40 cycles of 15 seconds at 95°C and 31 seconds at 58°C. Each sample was run in triplicate.

2.4. Construction of the soxR-overexpression strain

The full-length *soxR* gene was amplified by PCR from 1 CRAB isolate. The PCR product was ligated into pWH1266, which is an *Escherichia coli–Acinetobacter* shuttle plasmid,[10] using the In-Fusion HD Cloning kit (Clontech, Saint-Germain-en-Laye, France) and transformed into *E. coli* JM109 (TaKaRa). Plasmids were isolated from transformants and used to transform electrocompetent *A. baumannii*, which were selected on Mueller–Hinton agar containing 100 μg/mL ticarcillin.

2.5. Growth rate assay

Bacteria were inoculated into 5 mL of Mueller–Hinton broth and incubated overnight at 37°C. Overnight cultures of *A. baumannii* strains were diluted 1:100 in Mueller–Hinton broth, and growth curves were performed in triplicate by incubating the cultures for 24 hours at 37°C with shaking at 200 rpm. Bacterial growth was monitored by measuring the optical density of the culture at 620 nm.

2.6. In vitro competition assay

In vitro competition assays were performed as described previously.[11] Briefly, the *A. baumannii* wild-type strain and the *soxR*-overexpression strain were cultured separately overnight in LB broth at 37°C. The bacteria were diluted 1:100, and equivalent numbers of the wild-type strain and the *soxR*-overexpression strain were pooled and cultured together at 37°C. At 0, 4, and 24 hours, aliquots of the mixed bacteria were diluted with sterile phosphate-buffered saline and plated onto 2 LB agar plates, one of which contained 100 μg/mL ticarcillin. Colony-forming units (CFU) were counted after 24 hours of incubation at 37°C. The competitive index (CI) was determined as follows: CI = (*soxR*-overexpression CFU/wild-type CFU)/(*soxR*-overexpression CFU/inoculated wild-type CFU).

2.7. Statistical analysis

SPSS 17.0 for Windows (SPSS Inc, Chicago, IL) was used for all statistical analyses. Comparative analyses were executed by χ^2 or Fisher exact tests for categorical variables, and by the Student's *t* test for continuous variables. All tests were 2-tailed, and *P* values $<.05$ were considered statistically significant.

3. Results

3.1. Correlation between soxR mutations and antibiotic resistance in A. baumannii

To assess the correlation between antibiotic resistance and the global regulator SoxR, *soxR* mutations were investigated. In most CRAB isolates (29/32, 90.63%), a mutation in *soxR* (G115A) resulted in an amino acid substitution (Gly39Ser) in SoxR (Table 1). In 11 CSAB isolates, the Gly39Ser mutation was only identified in 1 strain, and the frequency (9.1%) was lower than that of the CRAB strains ($P<.01$). However, *soxS* was absent in all strains analyzed (Table 1).

3.2. Contribution of soxR overexpression to A. baumannii antibiotic resistance

To decipher the role of SoxR in *A. baumannii*, the *soxR*-overexpression mutant AB26/soxR was constructed. Susceptibility testing was performed with AB26/soxR, and the parental strain AB26 was used as a control (Table 2). The data showed that *soxR* overexpression resulted in increased susceptibility to chloramphenicol (4-fold), tetracycline (2-fold), tigecycline (2-fold), ciprofloxacin (2-fold), amikacin (2-fold), and trimethoprim (2-fold), but it did not influence imipenem susceptibility.

3.3. Influence of soxR overexpression on efflux pump gene expression

Multiple efflux pump genes, which correlate with antibiotic resistance in *A. baumannii*, including resistance-nodulation-division (RND) family genes (*adeB*, *adeJ*, and *adeG*), the small multidrug resistance (SMR) family gene *abeS*, the multidrug and toxic compound extrusion (MATE) gene *abeM*, and the major

Table 1

Correlation between *soxR* mutation and antibiotic resistance in *A. baumannii*.

Gene	Amino acid sequence analysis		No. of isolates		*P*
	Location	Amino acid	CRAB (n = 32)	CSAB (n = 11)	
soxR	39	Gly	3	10	<0.01
		Ser	29	1	
soxS		Absent	32	11	–

CRAB = carbapenem-resistant *Acinetobacter baumannii*, CSAB = carbapenem-susceptible *Acinetobacter baumannii*.

Table 2

Antibiotic susceptibility change of *soxR*-overexpression strain.

Antibiotics	Minimum inhibitory concentration (mg/L)		
	AB26	AB26/soxR	AB26 vs AB26/soxR
Chloramphenicol	96	24	4
Tetracycline	2	1	2
Tigecycline	0.125	0.064	2
Ciprofloxacin	0.125	0.064	2
Amikacin	4	2	2
Trimethoprim	8	4	2
Imipenem	0.25	0.25	1

AB26 = the wild-type strain, AB26/soxR = the *soxR*-overexpression strain.

facilitator superfamily gene *craA* were investigated. As shown in Figure 1, the expression of *abeS*, *abeM*, *adeJ*, and *adeG* was decreased in AB26/soxR with statistical significance ($P < .05$). The expression level of *abeS* was decreased 3.8-fold in *soxR*-overexpression isolates, which showed the most obvious down-regulation. However, no significant difference was detected in *craA* or *adeB* expression among *soxR*-overexpression isolates (data not shown).

3.4. Effect of SoxR on the growth and fitness of A. baumannii

To determine whether *soxR* overexpression affects the bacterial growth rate, growth curves were performed for the *soxR*-overexpression strain AB26/soxR and the wild-type strain AB26. However, the growth rate of AB26/soxR did not differ significantly from that of AB26. *In vitro* competition experiments were also performed to determine the relative growth rates of each of the strains (Fig. 2). The growth rate of AB26/soxR was lower than that of AB26 when cultured together. In addition, *soxR* overexpression was associated with a significant fitness cost *in vitro*. Measurements were performed at 4 and 24 hours, and a significant decrease in the growth of the AB26/soxR strain was observed. After a 4-hour co-culture, the CI was 0.723 ($P < .05$), and after a 24-hour co-culture, the CI was even lower (0.299) and highly significant ($P < .01$).

4. DISCUSSION

SoxR is a global repressor protein involved in multidrug-resistance in *Enterobacteriaceae*.[3,4] SoxR protein is produced constitutively at a low level, and it activates the expression of the *soxS* gene in response to superoxide-generating agents.[12] The activity of SoxS is regulated only by its intracellular concentration, and it binds promoter regions of its target genes to recruit RNA polymerase.[12] High *SoxS* activity is attributable to a frameshift mutation that truncates *soxR*, and it is responsible for fluoroquinolone resistance in *E. coli*.[13] In *Klebsiella pneumoniae*, a mutation in soxR (C375G) results in an amino acid substitution (N125K) in SoxR protein. The *soxR* mutation induced the overexpression of efflux systems, which were phenotypically characterized by multidrug resistance.[5] Recent studies of the SoxR regulon in *Pseudomonas* indicate that SoxR plays an alternative role to that in the traditional SoxR–SoxS paradigm. In *P. aeruginosa*, SoxR can directly activates a 6-gene regulon, which includes a multidrug efflux pump system encoded by the *mexGHI-ompD* 4-gene operon, and a probable efflux pump gene PA3718.[14] In our study, we identified a correlation between decreased antibiotic susceptibility and the frequency of clinical strains carrying the Gly39Ser mutation in *soxR*. However, the *soxS* gene was absent in all *A. baumannii* strains in our study. We speculate that SoxR may directly regulate target genes, such as efflux pump genes, in *A. baumannii*. Because *soxS* upregulation is associated with antibiotic resistance in

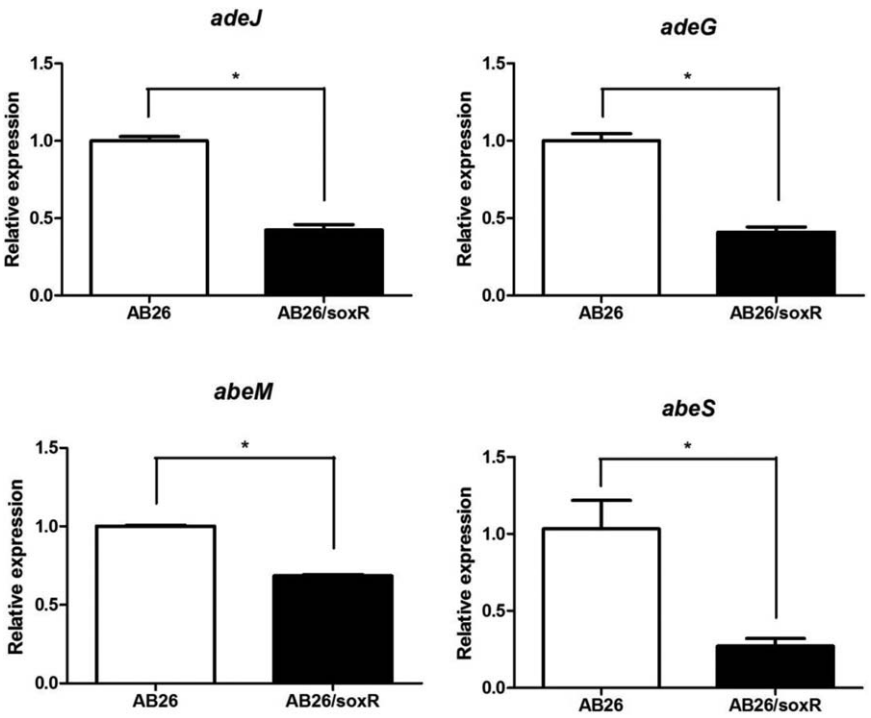

Figure 1. Relative expression of efflux pump genes in *A. baumannii*. *, $P < .05$. AB26, the wild-type strain; AB26/soxR, the *soxR*-overexpression strain.

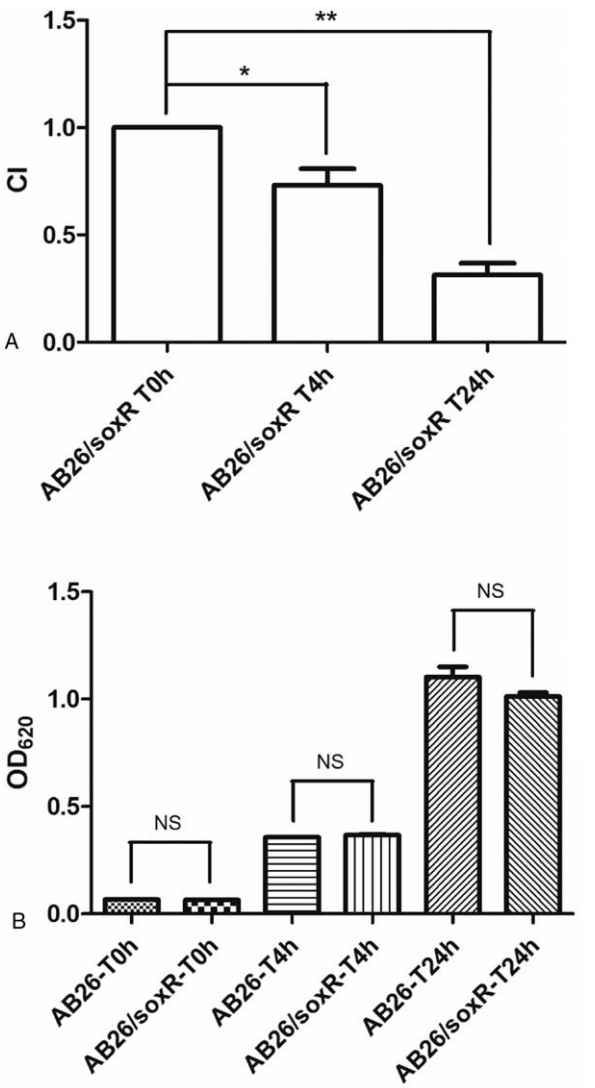

Figure 2. Relative *in vitro* competition indexes of the *soxR*-overexpression strain. (A) *In vitro* competition indexes at 4 and 24 hours. *, $P < .05$. **, $P < .01$. (B) Growth rate of the *soxR*-overexpression strain and the wild-type strain. AB26, the wild-type strain; AB26/*soxR*, the *soxR*-overexpression strain. NS = no significant difference.

Enterobacteriaceae, and *soxS* was absent in all *A. baumannii* strains in our study, a *soxR*-overexpression mutant was constructed to analyze the contribution of SoxR to antibiotic resistance and fitness in *A. baumannii*.

Efflux-mediated resistance has been found in many bacterial genera.[15] Overexpression of the efflux system, responsible for reducing the accumulation of antibiotics, is an efficient mechanism for multidrug resistance. RND efflux systems (AdeABC, AdeIJK, and AdeFGH), which confer multidrug resistance when overexpressed, typically exhibit wide substrate ranges.[15] The AbeM and AbeS pumps that belong to the MATE- and SMR-type exporters, respectively, also accommodate many drug substrates.[16] The deletion of the SMR family gene *abeS* resulted in significant decreases in the minimum inhibitory concentrations of chloramphenicol, ciprofloxacin, and erythromycin in *A. baumannii*.[17] In our study, we investigated the effect of overexpressing *soxR* on the expression of several efflux pump genes, including the RND family genes *adeB*, *adeJ*, and *adeG*; the SMR family gene *abeS*; the MATE family gene *abeM*; and the major facilitator superfamily gene *craA*. The expression levels of the RND genes *adeJ* and *adeG* were 2.4- and 2.5-fold lower in isolates with overexpression of *soxR*. Meanwhile, real-time RT-PCR showed that *abeS* expression was 3.8-fold lower in the *soxR*-overexpression strain. The change in expression of multiple efflux pump genes suggests that SoxR may be involved in a complex regulatory network.

The inability to construct a *soxR* deletion mutant is a limitation of our study. Construction of the *soxR* deletion mutant failed even though we successfully used the same method to construct an *adeB* deletion mutant.[18] We will attempt to construct the *soxR*-deletion mutant using new approaches in the future. Only studying the regulatory effects of SoxR on efflux pump genes is another limitation of our study. Studying other regulatory elements could increase our understanding of the role of SoxR in antibiotic resistance and fitness in *A. baumannii*. Future transcriptome analyses and functional studies are needed to fully understand the detailed regulatory mechanism of SoxR.

This is the first time, to our knowledge, that SoxR has been associated with the regulation of fitness and antibiotic resistance in *A. baumannii*. We found a correlation between decreased antibiotic susceptibility and the frequency of clinical strains carrying the Gly39Ser mutation in *soxR*. *SoxR* overexpression was associated with a significant fitness cost *in vitro*, and *soxR* overexpression correlated with the decreased expression of the efflux pump genes *abeS*, *abeM*, *adeJ*, and *adeG*. These results reveal that SoxR is a negative regulator of efflux pump gene expression and affects antibiotic resistance and fitness in *A. baumannii*.

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