DksA Modulates Antimicrobial Susceptibility of Acinetobacter baumannii

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Abstract: The stringent response regulators, (p)ppGpp and DksA, modulate various genes involved in physiological processes, virulence, and antimicrobial resistance in pathogenic bacteria. This study investigated the role of DksA in the antimicrobial susceptibility of Acinetobacter baumannii. The ΔdksA mutant (KM0248D) of A. baumannii ATCC 17978 and its complemented strain (KM0248C) were used, in addition to the ΔdksA mutant strain (NY0298D) of clinical 1656-2 strain. The microdilution assay was used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents. Quantitative real-time PCR was performed to analyze the expression of genes associated with efflux pumps. The KM0248D strain exhibited an increase of MICs to quinolones and tetracyclines, whereas KM0248D and NY0298D strains exhibited a decrease of MICs to aminoglycosides. The expression of genes associated with efflux pumps, including adeB, adeF, adeM, and/or tetA, was upregulated in both ΔdksA mutant strains. The deletion of dksA altered bacterial morphology in the clinical 1656-2 strain. In conclusion, DksA modulates the antimicrobial susceptibility of A. baumannii. The ΔdksA mutant strains of A. baumannii upregulate efflux pump gene expression, whereas (p)ppGpp-deficient mutants downregulate efflux pump gene expression. (p)ppGpp and DksA conduct opposite roles in the antimicrobial susceptibility of A. baumannii via efflux pump gene regulation.

Keywords: Acinetobacter baumannii; DksA; (p)ppGpp; antimicrobial susceptibility; efflux pump gene

1. Introduction

Acinetobacter baumannii is a notorious nosocomial pathogen causing various infections, including pneumonia, bloodstream infections, and urinary tract infections, in critically ill patients [1,2]. A. baumannii rapidly acquired drug-resistant determinants, such as Ambler class B metallo-β-lactamase genes and class D bιOXA genes, and the prevalence of carbapenem-resistant A. baumannii is a major concern worldwide [3,4]. In 2017, the World Health Organization proposed that carbapenem-resistant A. baumannii is the top priority pathogen for new antibiotic development [5]. Furthermore, under antibiotic selective pressure, this microorganism could develop resistance to commonly used antimicrobial agents by intrinsic resistance mechanisms, such as overexpression of efflux pump genes, permeability defects, and gene mutations that alter or modify target sites [3,6,7]. Of these resistance mechanisms, multiple efflux pumps play an important role in resistance to different classes of antimicrobial agents in A. baumannii [8–10]. The accumulation of acquired and intrinsic resistance mechanisms results in difficulty in the treatment of multidrug-resistant A. baumannii infections [11,12].

Bacterial alarmones, guanosine-5′,3′-tetraphosphate (pppGpp) and guanosine-5′,3′-pentaphosphate (pppGpp), collectively known as (p)ppGpp, are responsible for the bacterial stringent response by primarily regulating RNA polymerase (RNAP) activity [13,14]. DksA binds to the secondary channel of RNAP and allosterically modulates RNAP activity [15]. (p)ppGpp may work synergistically or independently with DksA [16]. The (p)ppGpp-deficient (ArelA ΔspoT) mutant was more susceptible to different classes of...
antimicrobial agents than the wild-type (WT) Escherichia coli strain [17]. Additionally, the ΔdksA mutant was more susceptible to antimicrobial agents, including β-lactams, aminoglycosides, quinolones, and tetracyclines, than the WT E. coli strain [18]. These results indicate that DksA and (p)ppGpp coordinately regulate the transcription of genes involved in antimicrobial resistance. There was no difference in the minimum inhibitory concentrations (MIC) of ciprofloxacin and ofloxacin between the WT and ΔdksA mutant strains of Pseudomonas aeruginosa, but the minimum bactericidal concentrations of quinolones increased in the ΔdksA mutants [19]. We recently demonstrated that (p)ppGpp-deficient (ΔA1S_0579) mutant was more susceptible to antimicrobial agents, including cephalosporins, monobactam, carbapenems, fluoroquinolones, aminoglycosides, colistin, tetracyclines, and trimethoprim, than the WT A. baumannii ATCC 17978 strain via down-regulation of various efflux pump genes [20]. However, the role of DksA in antimicrobial susceptibility has not been characterized in A. baumannii. This study investigated the role of DksA in the antimicrobial susceptibility of A. baumannii using WT A. baumannii, ΔdksA mutant, and dksA-complemented strains.

2. Results
2.1. The Effect of dksA on the Antimicrobial Susceptibility of A. baumannii ATCC 17978

To examine the role of DksA in antimicrobial susceptibility of A. baumannii ATCC 17978, the minimum inhibitory concentrations (MICs) of antimicrobial agents for WT, ΔdksA mutant (KM0248D), and dksA-complemented (KM0248C) strains were determined. Of the 15 antimicrobial agents tested, MICs of five agents, including quinolones (nalidixic acid, ciprofloxacin, and levofloxacin) and tetracyclines (tetracycline and tigecycline) increased more than two-fold in the ΔdksA mutant strain compared with the WT strain (Table 1). However, the MICs of aminoglycosides (amikacin, gentamicin, and tobramycin) decreased more than two-fold in the ΔdksA mutant strain compared with the WT strain. Quantitative real-time PCR (qPCR) was conducted to determine whether efflux pump genes were responsible for the changes in the MICs of antimicrobial agents against the ΔdksA mutant strain. The expression of efflux pump genes, including adeB, adeI, and adeJ for resistance nodulation cell division (RND)-type multidrug efflux pumps, tetA for a major facilitator superfamily (MFS)-type drug efflux transporter, and abeM for a multidrug and toxic compound extrusion (MATE)-type multidrug efflux transporter, was significantly increased in the ΔdksA mutant strain compared with the WT A. baumannii ATCC 17978 strain (Figure 1A). However, the expression of the adeI and adeJ in the dksA-complemented strain was not restored compared to the WT strain.

**Table 1.** Antimicrobial susceptibility of wild-type A. baumannii, ΔdksA mutant, and dksA-complemented strains.

| Antibacterial Agents | MIC (µg/mL) | Fold Change (KM0248D/WT) | MIC (µg/mL) | Fold Change (NY0298D/WT) |
|----------------------|-------------|--------------------------|-------------|--------------------------|
|                      | ATCC 17978  | KM0248D                  | KM0248C     | 1656-2                   | NY0298D                  |
| Nalidixic acid       | 4           | 16                       | 8           | >256                     | >256                     |
| Ciprofloxacin        | 0.125       | 0.5                      | 0.125       | 2                        | 4                        |
| Levofloxacin         | 0.063       | 0.125                    | 0.063       | 2                        | 16                       |
| Cefoxitin            | 128         | 128                      | 128         | 1                        | >256                     |
| Cefotaxime           | 16          | 16                       | 16          | >256                     | >256                     |
| Ceftazidime          | 4           | 4                        | 4           | >256                     | >256                     |
| Imipenem             | 0.125       | 0.125                    | 0.125       | 1                        | 16                       |
| Meropenem            | 0.25        | 0.25                     | 0.25        | 1                        | 32                       |
| Amikacin             | 1.0         | 0.25                     | 0.5         | 0.25                     | 64                       |
| Gentamicin           | 0.5         | 0.25                     | 0.5         | 0.5                      | 256                      |
| Tobramycin           | 0.5         | 0.125                    | 0.25        | 0.25                     | 128                      |
| Tetracycline         | 1           | 2                        | 2           | 2                        | 32                       |
| Tigecycline          | 0.125       | 0.5                      | 0.125       | 4                        | 1                        |
| Colistin             | 2           | 2                        | 2           | 2                        | 1                        |
| Trimethoprim         | >32         | >32                      | >32         | 1                        | 32                       |
2.2. The Effect of dksA on the Antimicrobial Susceptibility and Cellular Morphology of a Clinical A. baumannii Strain

To examine the role of DksA in the antimicrobial susceptibility and cellular morphology of a clinical A. baumannii strain, ΔdksA mutant (NY0298D) of the clinical 1656-2 strain was constructed (Supplementary Figure S1A). Deletion of dksA in A. baumannii 1656-2 was confirmed by PCR analysis (Supplementary Figure S1B). The expression of dksA was not observed in the ΔdksA mutant strain (Supplementary Figure S1C). Additionally, we determined whether dksA deletion changed the antimicrobial susceptibility of the clinical A. baumannii 1656-2 strain. No difference was observed in the MICs of quinolones and tetracyclines between WT and NY0298D strains. However, the NY0298D strain exhibited increased susceptibility to aminoglycosides (amikacin, gentamicin, and tobramycin) like the ΔdksA mutant strain of A. baumannii ATCC 17978 (Table 1). The expression of efflux pump genes, including adeB, adeI, adeJ, and abeM, significantly increased in the ΔdksA mutant strain, compared with that in the WT strain (Figure 1B). The ΔdksA mutant NY0298D strain displayed more morphological heterogeneity than the WT strain at an optical density.
of 600 nm (OD$_{600}$) of 0.95–1.05 to 1.75–1.85 (Figure 2). These results suggest that \textit{dksA} deletion in the clinical 1656-2 strain increases efflux pump gene expression and alters bacterial morphology.

![Figure 2](image-url) A morphological difference between \textit{A. baumannii} 1656-2 and its \textit{ΔdksA} mutant NY0298D strains. Bacteria were cultured in LB with shaking to reach the indicated OD$_{600}$ and stained with Gram’s reagents. Bacterial morphology was observed using a light microscope. Magnification, 1000×.

### 3. Discussion

The (p)ppGpp-deficient and \textit{ΔdksA} mutants of \textit{E. coli} exhibit increased susceptibility to antimicrobial agents [17,18], implying that (p)ppGpp and DksA contribute to antimicrobial resistance in \textit{E. coli}. The (p)ppGpp-deficient mutant of \textit{A. baumannii} ATCC 17978 also exhibited increased susceptibility to antimicrobial agents [20]. However, in the present study, \textit{ΔdksA} mutant of \textit{A. baumannii} ATCC 17978 exhibited decreased susceptibility to quinolones and tetracyclines, whereas \textit{ΔdksA} mutants of \textit{A. baumannii} ATCC 17978 and 1656-2 exhibited increased susceptibility to aminoglycosides.

The deletion of \textit{dksA} upregulated the expression of \textit{adeB, adel, adef, abeM} and/or \textit{tetA} in \textit{A. baumannii} ATCC 17978 and the clinical 1656-2 strain. \textit{A. baumannii} ATCC 17978 was susceptible to quinolones and tetracyclines, whereas the clinical 1656-2 strain was resistant to quinolones and tetracycline and susceptible to tigecycline [21]. In the 1656-2 strain, resistance to quinolones was mediated by the mutations in the quinolone-resistance determining region of \textit{gyrA}, and resistance to tetracyclines was potentially mediated by several efflux pump genes [22]. Furthermore, multidrug-resistant \textit{A. baumannii} strains decrease cell envelope permeability against antimicrobial agents [23]. Therefore, the upregulation of efflux pump genes directly contributed to increased MICs of quinolones and tetracyclines in the \textit{ΔdksA} mutant of ATCC 17978, although efflux pump gene upregulation could not change the MICs of quinolones and tetracyclines in \textit{ΔdksA} mutant of 1656-2. Both \textit{ΔdksA} mutants of ATCC 17978 and 1656-2 were more susceptible to aminoglycosides than the WT strains. In a previous study, the (p)ppGpp-deficient strain of \textit{A. baumannii} ATCC 17978 was more susceptible to aminoglycosides than the WT strain [20]. Because (p)ppGpp and DksA inhibit the transcription of genes involved in the synthesis of translational machinery during the stringent response or stressful conditions [15,24], (p)ppGpp-deficient and \textit{ΔdksA} mutants cannot inhibit the transcription of ribosomal genes, potentially increasing susceptibility to aminoglycosides. Combined with the previous results, the present study demonstrates that (p)ppGpp and DksA play an opposing role in the regulation of genes...
associated with efflux pumps. Further studies would be required to understand the regulatory mechanisms of multiple genes linked with intrinsic resistance by DksA and (p)ppGpp in A. baumannii.

The present study demonstrated that ΔdksA mutants of clinical 1656-2 exhibited more morphological heterogeneity than the WT strain. Previous studies have reported that ΔdksA mutant and (p)ppGpp-deficient mutant strains exhibited more morphological heterogeneity than the WT A. baumannii ATCC 17978 strain [20,25]. The (p)ppGpp-deficient and ΔdksA mutants in E. coli also exhibited a more filamentous morphology than the WT strain [16]. These results indicate that (p)ppGpp and DksA coordinate regulatory mechanisms of the genes associated with cellular morphology or cell division.

This study demonstrates that dksA deletion upregulates efflux pump gene expression in A. baumannii strains. However, (p)ppGpp deficiency downregulates the expression of efflux pump genes in A. baumannii [16]. Overall, RNAP-binding global regulators (p)ppGpp and DksA can modulate antimicrobial susceptibility in A. baumannii, but they play opposite roles in antimicrobial resistance through regulating the efflux pump genes.

4. Materials and Methods

4.1. Bacterial Strains

Bacteria, including WT, ΔdksA mutant, and dksA-complemented strains, and plasmids used in this study are listed in Table 2. A. baumannii and E. coli strains were cultured in lysogeny broth (LB) (BioShop, Burlington, ON, Canada) at 37 °C. Mutant strains were selected in LB media containing chloramphenicol (20 µg/mL) or erythromycin (30 µg/mL).

Table 2. Bacterial strains and plasmids used in this study.

| Bacteria/Plasmids | Relevant Characteristics | Reference of Source |
|-------------------|--------------------------|---------------------|
| A. baumannii       |                          |                     |
| ATCC 17978         | Wild-type strain         | ATCC                |
| KM0248D           | ΔA1S_0248 of A. baumannii ATCC 17978 | [25]  |
| KM0248C           | A1S_0248 with T1 terminator in KM0248D | [25]  |
| 1656-2            | Clinical isolate         | [21]  |
| NY0298D           | ΔABK1_0298 of A. baumannii 1656-2 | This study |
| Plasmids          | pDM4                     | GenBank accession no. KC795686 |
|                   | pWH1266 with armA coding region and its promoter less nptI, and origin of replication with ermAM; KmG; Ery   |

Abbreviations: CmG, chloramphenicol-resistant; KmG, Kanamycin-resistant; EryG, erythromycin-resistant.

4.2. Construction of the ΔdksA Mutant of 1656-2 Strain

The ΔABK1_0298 gene of clinical A. baumannii 1656-2 strain, corresponding to the A1S_0248 gene of A. baumannii ATCC 17978, was deleted by a markerless gene deletion method [27]. Genomic DNAs purified from A. baumannii 1656-2 and pFL02 were used as polymerase chain reaction templates for the amplification of dksA and erythromycin resistance cassettes, respectively. The upstream and downstream regions of dksA were combined with an erythromycin resistance cassette through overlap extension PCR using specific primers with a ProFlex PCR system (Applied Biosystems, Foster City, CA, USA) (Supplementary Table S1). This mutated DNA fragment was ligated into Apal-digested pDM4. The pDM4 carrying the mutated DNA fragment was inserted into the chromosome of A. baumannii 1656-2 strain by transformation using Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA) and homologous recombination (Supplementary Figure S1A). The ΔdksA mutant of A. baumannii 1656-2 was named NY0298D (Table 2).
4.3. Antimicrobial Susceptibility Testing

The MICs of antimicrobial agents were determined by the microdilution method according to the Clinical Laboratory Standards Institute (CLSI) [28]. Antimicrobial agents included aminoglycosides (amikacin, gentamicin, and tobramycin), carbapenems (imipenem and meropenem), cephalosporins (ceftazidime, cefoxitin, and cefotaxime), quinolones (nalidixic acid, ciprofloxacin, and levofloxacin), tetracyclines (tetracycline and tigecycline), colistin and trimethoprim. E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as quality control strains.

4.4. RNA Isolation and qPCR

Bacteria were cultured in LB under shaking conditions for 18 h to analyze the efflux pump gene expression. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was conducted to synthesize cDNA using 1.5 µg of total RNA, random hexamer primers, and TOPscript reverse transcriptase (Enzymomics, Daejeon, Korea). The specific primers for efflux pump genes are listed in Supplementary Table S2. Gene transcripts were quantified using TOPreal qPCR 2X PreMIX (SYBR Green with high ROX) (Enzymomics) with a StepOnePlus Real-Time PCR Systems (Applied Biosystems). Melting curve analysis was conducted to evaluate the amplification specificity. The expression of efflux pump genes was normalized to the expression of the 16S rRNA gene, and the fold change was determined. Gene expression assays were performed in three independent experiments.

4.5. Gram Staining

A. baumannii strains were cultured overnight before being diluted to an OD$_{600}$ of 1.0. The bacterial samples were diluted 1:20 in fresh LB and cultured in LB under shaking conditions to reach the indicated OD$_{600}$. Bacteria were stained by Gram reagents (YD Diagnotics, Gyeonggi, Korea) [29] and then observed under a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan).

4.6. Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 software (San Diego, CA, USA). Data from different experimental groups were analyzed using one-way ANOVA with Dunnett’s post hoc analysis or Student’s t-test. Differences of $p < 0.05$ were considered statistically significant.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10121472/s1, Figure S1: Construction of the $\Delta$dksA mutant strain, Table S1: Primers used for the DNA cloning in this study, Table S2: Primers used for qPCR in this study.

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