Global regulator DksA modulates virulence of Acinetobacter baumannii

Nayeong Kim, Joo Hee Son, Kyeongmin Kim, Hyo Jeong Kim, Yoo Jeong Kim, Minsang Shin, and Je Chul Lee

Department of Microbiology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea

ABSTRACT
DksA with (p)ppGpp regulates a wide range of gene transcriptions during the stringent response. The aim of this study was to identify a DksA ortholog in Acinetobacter baumannii and clarify the roles of DksA in bacterial physiology and virulence. The ΔdksA mutant and its complemented strains were constructed using A. baumannii ATCC 17978. The A5S_0248 in A. baumannii ATCC 17978 was identified to dksA using sequence homology, protein structure prediction, and gene expression patterns under different culture conditions. The ΔdksA mutant strain showed a filamentous morphology compared with the wild-type (WT) strain. Bacterial growth was decreased in the ΔdksA mutant strain under static conditions. Surface motility was decreased in the ΔdksA mutant strain compared with the WT strain. In contrast, biofilm formation was increased and biofilm-associated genes, such as bfmR/S and csuc/D/E, were upregulated in the ΔdksA mutant strain. The ΔdksA mutant strain produced less autoinducers than the WT strain. The expression of abal and abar was significantly decreased in the ΔdksA mutant strain. Furthermore, the ΔdksA mutant strain showed less bacterial burden and milder histopathological changes in the lungs of mice than the WT strain. Mice survival was also significantly different between the ΔdksA mutant and WT strains. Conclusively, DksA is directly or indirectly involved in regulating a wide range of genes associated with bacterial physiology and virulence, which contributes to the pathogenesis of A. baumannii. Thus, DksA is a potential anti-virulence target for A. baumannii infection.

Introduction
Acinetobacter baumannii is an important opportunistic pathogen that causes a hospital-acquired infection in severely ill patients [1,2]. Current therapeutic options for A. baumannii infections are limited due to its antimicrobial resistance, posing a significant threat to global public health [3,4]. The World Health Organization declared carbapenem-resistant A. baumannii as a top priority pathogen for investing in new therapeutic drugs [5]. A. baumannii is considered to be a low virulent pathogen [1,6], but several virulence factors, including outer membrane proteins [7-9], phospholipases [10], lipopolysaccharides [11], protein secretion systems [12], capsular polysaccharides [13], and the acquisition and utilization of metal ions [14,15], have been identified in this microorganism. The ability to survive and persist in harsh environments or stressful conditions is also responsible for the pathogenicity of A. baumannii [1,16]. Two A. baumannii strains that showed the same pulsotype but produced different levels of exopolysaccharides exhibited different clinical scores and mortalities in a mouse pneumonia model [17]. This suggests that different regulation of virulence factors directly contributes to clinical outcomes. Understanding global regulatory mechanisms of virulence factors is required to identify new therapeutic targets for drug-resistant A. baumannii infections.

The stringent response is a global regulatory mechanism of genes that adapts bacteria to many stress conditions [18,19]. The production of signaling molecules guanosine-5′,3′-pentaphosphate (pppGpp) and guanosine-5′,3′-tetraphosphate (pppGpp), collectively known as (p)ppGpp, is the hallmark of this response [19,20]. (p)ppGpp interacts with two binding sites of RNA polymerase (RNAP), which is either activated or repressed in a promoter-dependent way, changing the expression of genes in response to various environmental stresses [21-23]. Binding of (p)ppGpp to the interface of ω and β′ subunits induces a conformational change of RNAP and influences the lifetime of the open complex [22,23]. Binding of (p)ppGpp to the interface of the secondary channel of β′ subunits influences the transcription initiation [23]. The DnaK suppressor protein (DksA), an RNAP-binding...
transcription factor of the stringent response, binds to the secondary channel of RNAP, which modulates the RNAP activity [24,25]. In *Escherichia coli*, (p)ppGpp and DksA function synergistically to down-regulate the transcription of genes associated with the synthesis of translational machinery and upregulate genes associated with the stress tolerance during the stringent response [24,26]. In addition to coordinated gene regulation, (p)ppGpp and DksA regulate gene expression independently or sometimes conduct an opposing role [27]. The small cytosolic protein DksA controls various cellular physiological processes, including amino acid biosynthesis [26], translation [24], DNA repair [28,29], cellular division [30], and oxidative stress responses [31] in Gram-negative bacteria. DksA has been reported to regulate virulence and antimicrobial resistance in Gram-negative pathogens [32–35]. Thus, DksA may work independently or along with (p)ppGpp to affect the RNAP activity, contributing to bacterial physiology, pathogenesis, and antimicrobial resistance.

In *A. baumannii*, RelA, a (p)ppGpp synthetase, has been recently characterized. ΔrelA mutant exhibits hypermotility, cell elongation, and low virulence in the *Galleria mellonella* infection model [36]. Furthermore, (p)ppGpp-deficient mutants are more susceptible to antimicrobial agents, including gentamicin, tetracycline, erythromycin, and trimethoprim, than wild-type (WT) *A. baumannii* ATCC 17978 strain by downregulating efflux pump genes [37]. Although the functional characterization of (p)ppGpp in *A. baumannii* has been made, DksA has not been identified. Therefore, this study identified a DksA ortholog in *A. baumannii* and clarified the roles of DksA in bacterial physiology and virulence using WT *A. baumannii* ATCC 17978, ΔdksA mutant, and dksA-complemented strains.

### Materials and methods

#### Bacterial strains

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *A. baumannii* and *E. coli* strains were grown in lysogeny broth (LB) at 37°C. To select colonies during the construction of mutant and complementary strains, antimicrobial agents, chloramphenicol (20 μg/ml) or kanamycin (50 μg/ml), were added to the growth medium.

#### Sequence homology and prediction of protein structure

Nucleotide sequences of AIS_0248 were obtained from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Multiple alignments of the translated sequences of AIS_0248 with DksA of other bacterial species were performed using the constraint-based alignment tool for multiple protein sequences (COBALT) (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi). WebLogo 3 was used to identify the sequence logo between *E. coli* DksA and the translated sequences of AIS_0248 of *A. baumannii* ATCC 17978 (http://weblogo.threeplu sone.com/). The three-dimensional protein structure of the AIS_0248 gene product was predicted using SWISS-MODEL (https://swissmodel.expasy.org/).

#### Construction of the ΔdksA mutant and dksA-complemented strains

The ΔAIS_0248 mutant strain (KM0248D) was constructed using *A. baumannii* ATCC 17978 by a markerless gene deletion method as previously described [38]. The complementation of AIS_0248 in the KM0248D strain was conducted using an overlap extension polymerase chain reaction (PCR) and the AIS_0248-complemented strain (KM0248C) was constructed. The specific PCR primers used to construct the mutant and complemented strains are listed in Supplementary Table S2. The construction of mutant and complemented strains is presented in Supplementary Figure S1 and the Supplementary Materials and methods section in detail.

#### Bacterial growth curve and gram staining

*A. baumannii* strains were cultured overnight in LB and then diluted to an optical density at 600 nm (OD\textsubscript{600}) of 1.0. The bacterial suspension was diluted at 1:20 in fresh LB and cultured under shaking and static conditions for 24 and 48 h at 37°C, respectively. Bacterial growth was measured at OD\textsubscript{600} using a spectrophotometer (Biochrom, Cambridge, UK). A bacterial growth assay was performed in two independent experiments. *A. baumannii* strains were cultured on M9 agar plates to determine the growth of bacteria in minimal media. For Gram staining, bacteria were grown in LB under shaking conditions to reach the indicated OD\textsubscript{600}. Bacterial samples were stained by
Gram’s reagents and observed using the light microscope (Eclipse E600, Nikon, Tokyo, Japan).

**RNA isolation and quantitative real-time PCR (qPCR)**

*A. baumannii* ATCC 17978 was cultured in LB under static or shaking conditions at 23, 30, or 37°C for 18 h to analyze the expression of *dksA* under different culture conditions. *A. baumannii* strains were cultured in LB under static or shaking conditions for 18 or 24 h to analyze the expression of virulence-associated genes. Total RNA was isolated from *A. baumannii* strains using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (1.5 µg) was reverse-transcribed to synthesize cDNA using random hexamer primers and TOPOscript™ reverse transcriptase (Enzymics, Daejeon, Korea). The specific primers for target genes are listed in Supplementary Table S3. Gene transcripts were quantified using TOPOreal™ qPCR 2X PreMIX (SYBR Green with high ROX) (Enzymics) with a StepOnePlus™ Real-time PCR Systems (Applied Biosystems, Foster City, CA, USA). The fold change in the expression level of each gene was calculated by the ∆∆Ct method using the 16S rRNA gene as an internal control. The expression of genes was performed in two or three independent experiments.

**Surface motility assay**

*A. baumannii* strains were cultured in LB under shaking conditions overnight. Bacterial cultures were diluted in LB to an OD₆₀₀ of 1.0, and bacterial suspension (2 µl) was inoculated onto motility agar plates containing tryptone 1%, yeast extract 0.5%, and Eiken agar powder 0.3% [39]. The diameters of bacterial migration were measured at 4, 7, and 12 h. The surface motility assay was performed in three independent experiments.

**Biofilm formation assay**

For biofilm formation assay of *A. baumannii* strains, LB broth without sodium chloride was used. Bacteria were cultured overnight in LB and then diluted to an OD₆₀₀ of 1.0. The bacterial suspension (2 ml) diluted with 1:40 in fresh LB was inoculated into wells of a 24-well plate and incubated without shaking for 10, 24, and 48 h. After removing liquid media containing the planktonic cells, biofilm cells were washed with phosphate-buffered saline (PBS) and stained with a 0.1% (w/v) crystal violet solution for 15 min. The biofilm cell-associated dye was eluted with 95% ethanol and absorbance of the eluted solvent was measured at OD₅₇₀. Biofilm mass at OD₅₇₀ was normalized to bacterial growth at OD₆₀₀. The assay was performed in three independent experiments.

**Bioassay for detecting quorum sensing molecules**

The reporter strain *Agrobacterium tumefaciens* NT1 (pDCI141E33) was cultured in the defined minimal medium at 30°C [40]. *A. baumannii* strains were cultured in LB overnight at 37°C and then adjusted to an OD₆₀₀ of 1.0. Bacterial samples (5 µl) were spotted onto the bioassay plate overlaid with the reporter strain [41]. As a control, N-(3-hydroxy-dodecanoyl)-DL-homoserine lactone (Sigma-Aldrich, St. Louis, MO, USA) was used. The blue zone was measured after the plate was incubated at 30°C for 18 h. The bioassay was performed in three independent experiments.

**Animal experiments**

Female BALB/c mice (eight-week-old) were kept under conventional conditions. Cyclophosphamide was used to induce neutropenia in mice as previously described [42]. Neutropenic mice were intraperitoneally infected with 5 × 10⁸ colony forming units (CFUs) of the *A. baumannii* strains, and the survival of mice was evaluated for 5 days after bacterial infection. Neutropenic mice were infected with 5 × 10⁷ CFUs of the *A. baumannii* strains intratracheally to evaluate bacterial loads and histological changes in the lungs. The surviving mice were sacrificed 24 h post-infection; also, cardiac puncture was performed to obtain blood samples, and both lungs were removed. The CFUs in blood and right lung homogenates were determined on LB agar plates. Left lung tissues were fixed in formalin solution and stained with hematoxylin and eosin. All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Kyungpook National University.

**Statistical analysis**

Data were analyzed by one-way analysis of variance with Dunnett’s post hoc analysis and Student’s t-tests using GraphPad Prism 5.0 (San Diego, CA, USA). Differences at *p* < 0.05 were considered statistically significant.
Results

Identification of dksA in A. baumannii

The A1S_0248 gene (537 bases) of A. baumannii ATCC 17978 was predicted to be dksA at NCBI, but the protein encoded by A1S_0248 had not been characterized. The homology of translated sequences of A1S_0248 ranged from 28.1% in the DksA of Streptococcus pyogenes (106 amino acids) to 51.7% in the DksA of E. coli (151 amino acids) (Figure 1(a)). The sequence logo showed that all sequences required for RNAP binding and DksA activity were identical, except for RNAP binding and DksA activity.

Figure 1. Identification of DksA in A. baumannii. (a) Multiple alignments of translated sequences of A1S_0248 with DksA of other bacterial species. The gray highlight indicates identical amino acids with E. coli DksA. The bold characters indicate >50% consensus amino acids among the tested bacterial species. Sequence conservation is presented as the height of colored bars. The accession number of DksA proteins is ABO10723 in A. baumannii ATCC 17978, VWQ00927 in E. coli, CDO15944 in Klebsiella pneumoniae, WP_058139749 in Pseudomonas aeruginosa, KIS47657 in Burkholderia cepacia, RTY79203 in Staphylococcus aureus, WP_047919748 in Neisseria gonorrhoeae, WP_148842572 in Streptococcus pyogenes, SGC70216 in Mycobacterium tuberculosis, and VTQ30573 in Streptococcus pneumoniae. (b) Sequence logo of DksA between E. coli and A. baumannii ATCC 17978 using the WebLogo 3. The red boxes are essential amino acids for RNAP binding and DksA activity in E. coli. The number is based on the amino acid sequences of E. coli DksA. Furthermore, 151 amino acids of E. coli DksA (the first number in parenthesis) are compared with the translated amino acid sequences of A1S_0248 of A. baumannii ATCC 17978 (the second number in parenthesis) in the boxes as follows: D (74, 101); A (76, 103); R (91, 118); R (125, 152); A (128, 155); I (136, 163); E (143, 170); I-M (144–149, 171–176). (c) The predicted three-dimensional structure of proteins encoded by A1S_0248 using Swiss-MODEL. Red and green colors indicate the amino acids for the RNAP binding and DksA activity, respectively.
for R145 and M149 in *E. coli* DksA, between the A1S_0248 gene product and *E. coli* DksA [25] (Figure 1(b)). The protein structure of A1S_0248 obtained using the SWISS-MODEL showed a high structural similarity with *E. coli* DksA [23] (Figure 1(c)). Next, to determine whether A1S_0248 was responsible for the stringent response, *A. baumannii* ATCC 17978, ΔA1S_0248 mutant, and A1S_0248-complemented strains were cultured on M9 minimal media. WT and A1S_0248-complemented strains were grown on LB and M9 agar plates, but the ΔA1S_0248 mutant exhibited no growth on M9 minimal media (Figure 2(a)). *A. baumannii* ATCC 17978 was cultured under different culture conditions, and the expression of A1S_0248 was analyzed using qPCR. The expression of A1S_0248 was upregulated more than 6-folds in bacteria cultured under static conditions than shaking conditions (Figure 2(b)). The expression of A1S_0248 was higher in bacteria cultured at 23°C (Figure 2(c)). Based on the sequence homology, protein structure prediction, bacterial growth on minimal media, and gene expression patterns under different culture conditions, A1S_0248 will hereafter be referred to as dksA.

**Construction of ΔdksA mutant and dksA-complemented strains**

To investigate the roles of *A. baumannii* DksA in the bacterial physiology and virulence, ΔdksA mutant (KM0248D) and dksA-complemented (KM0248C) strains were constructed using *A. baumannii* ATCC 17978 (Supplementary Figure S1(a) and S1(b)). PCR

---

**Figure 2.** Growth of *A. baumannii* strains in minimal media and the expression of dksA in *A. baumannii* ATCC 17978 under different culture conditions. (a) WT *A. baumannii* ATCC 17978 (1), ΔdksA mutant (2), and dksA-complemented (3) strains were grown on LB and M9 agar plates for 24 h. (b) and (c) The expression of dksA in *A. baumannii* ATCC 17978 was analyzed using qPCR. (b) Bacteria were cultured in LB under shaking or static conditions for 18 h. (c) Bacteria were cultured in LB under shaking at 23, 30, or 37°C for 18 h. The data are presented as the mean ± SD of three independent experiments. *** p < 0.001.
analysis confirmed the deletion and complementation of dksA (Supplementary Figure S1(c)). qPCR was performed to analyze the expression of dksA in WT, ΔdksA mutant, and complemented strains. The expression of dksA was observed in WT and dksA-complemented strains but not in ΔdksA mutant strain (Supplementary Figure S1(d)).

**Differences in cell morphology and growth kinetics between WT and ΔdksA mutant strains**

To assess whether the deletion of dksA could affect bacterial morphology, such as (p)ppGpp-deficient mutant [36,37], *A. baumannii* strains were cultured in LB to reach the indicated OD<sub>600</sub>, and bacterial morphology was examined by Gram staining. The ΔdksA mutant showed more filamentous morphology than WT and dksA-complemented strains at an OD<sub>600</sub> of 0.95–1.05 and 1.35–1.45. In contrast, the ΔdksA mutant showed similar morphology at OD<sub>600</sub> of 1.85–1.95 compared with WT and dksA-complemented strains (Figure 3(a)). To determine whether the deletion of dksA could affect growth of *A. baumannii*, bacteria were cultured in LB under static or shaking conditions, and bacterial growth was measured at OD<sub>600</sub>. Bacterial growth was similar among the WT, ΔdksA mutant, and

![Figure 3. Cellular morphology and growth kinetics of *A. baumannii* strains. (a) WT, ΔdksA mutant (KM0248D), and dksA-complemented (KM0248C) strains were cultured in LB under shaking conditions to reach the indicated OD<sub>600</sub> and stained by Gram staining. Bacterial morphology was observed using a light microscope. Magnification, 1,000X. (b) *A. baumannii* strains were cultured in LB under shaking conditions for 24 h and LB under static conditions for 48 h. Bacterial growth was measured at OD<sub>600</sub>. The result is representative of two independent experiments.](image-url)
dksA-complemented strains under shaking conditions, although the WT strain exhibited a higher OD_{600} than ΔdksA mutant and dksA-complemented strains (Figure 3(b)). However, a significant growth retardation of ΔdksA mutant under static conditions was observed, although WT and dksA-complemented strains showed different growth rates (Figure 3(b)). These results suggest that the deletion of dksA alters cellular morphology and growth kinetics under static conditions in A. baumannii ATCC 17978.

**Differences in surface motility and biofilm formation between WT and ΔdksA mutant strains**

To determine whether the deletion of dksA could affect surface motility of A. baumannii, bacteria were cultured in the motility agar plates, and migration of bacteria into the agar plates was measured. The ΔdksA mutant strain exhibited significantly smaller migration diameters than the WT strain at all tested times (Figure 4). The surface motility of dksA-complemented strain was partially restored at 7 h, but was fully restored at 12 h. Next, to assess whether the deletion of dksA could affect biofilm formation of A. baumannii, bacteria were cultured in 24-well plates for 48 h, and biofilm mass was measured. In contrast to the surface motility, ΔdksA mutant produced significantly more biofilm mass than WT and dksA-complemented strains (Figure 5). The difference in biofilm mass between WT and ΔdksA mutant strains was prominent at 48 h. To understand the regulation of biofilm-associated genes by DksA, the expression of bfmR/S for the two-component system, csuC/D/E for the chaperone-usher pilus system, and ompA for outer membrane protein A was analyzed in WT, ΔdksA mutant, and dksA-complemented strains cultured under static conditions for 24 h using qPCR. The expression of bfmR/S and csuC/D/E was significantly upregulated in the ΔdksA mutant strain, whereas the ompA expression was significantly downregulated in

![Figure 4](image-url). Surface motility of A. baumannii strains. WT A. baumannii ATCC 17978, ΔdksA mutant (KM0248D), and dksA-complemented (KM0248C) strains were cultured in motility agar plates for 4, 7, and 12 h, and the diameters (mm) of bacterial migration on agar plates were measured. Data are presented as the mean ± SD of three independent experiments. *** p < 0.001 compared with the WT strain.
the ∆dksA mutant strain (Figure 6). These results suggest that DksA modulates surface motility and biofilm formation of A. baumannii ATCC 17978.

Differences in the production of autoinducers between WT and ∆dksA mutant strains

To determine whether the deletion of dksA could affect the quorum sensing systems of A. baumannii, three A. baumannii strains were spotted onto plates overlaid with the reporter strain, and autoinducer production was determined. The ∆dksA mutant produced a smaller blue zone than WT and dksA-complemented strains (Figure 7(a)). The expression of abaI and abaR was significantly decreased in the ∆dksA mutant than the WT strain (Figure 7(b)). These results suggest that DksA regulates quorum sensing systems in A. baumannii ATCC 17978.

Effect of DksA on mice infected with A. baumannii strains

Neutropenic mice were intraperitoneally infected with A. baumannii strains, and their survival was monitored for five days. All mice infected with WT and dksA-complemented strains died within two
days, whereas two (28.6%) of seven mice infected with the ΔdksA mutant survived (Figure 8(a)). Seven neutropenic mice in each group were infected with A. baumannii strains intratracheally to assess whether deleting dksA affects the pathogenesis of A. baumannii. Bacterial burden in the lungs and blood of surviving mice was determined 24 h after bacterial injection. Three, five, and four mice infected with WT, ΔdksA mutant, and dksA-complemented strains survived at 24 h post-infection, respectively. The CFUs in the lungs showed a difference of >1,000-folds between WT and ΔdksA mutant strains (Figure 8(b)). The mean value of CFUs in the blood was slightly decreased in the mice infected with the ΔdksA mutant strain, but significant difference was not observed between the two groups. WT and dksA-complemented strains induced severe lung pathologies, such as destruction of alveoli, congestion, and exudates, whereas mild pathological events were observed in the lungs of mice infected with the ΔdksA mutant strain (Figure 8(c)). These results suggest that DksA directly contributes to the pathogenesis of A. baumannii ATCC 17978 in vivo.

**Discussion**

The present study demonstrates that A. baumannii DksA plays a role in bacterial morphology and growth under static conditions. Furthermore, A. baumannii DksA modulates biofilm formation, surface motility, and production of autoinducers in vitro and contributes to the pathogenesis in vivo. Overall, A. baumannii DksA plays multifaceted roles in bacterial physiology and virulence.

We first tried identifying a DksA ortholog in A. baumannii. The AIS_0248 gene in A. baumannii ATCC 17978 was predicted to be dksA at NCBI. The translated sequences of AIS_0248 were conserved among the predicted dksA of the sequenced A. baumannii strains (data not shown) but were different from E. coli DksA in size and amino acid sequences. In E. coli DksA, R125, A128, I136, and E143 and I144-
M149 are critical residues interacting with the β’ rim helices and βSI1 of RNAP, respectively [25]. D74, A76, and R91 are essential residues for DksA activity [25]. These amino acids essential for RNAP binding and DksA activity were conserved in the A1S_0248 gene products, except for K145 and N149. Additionally, structural analysis predicted that A1S_0248-encoded proteins carried zinc-binding, coiled-coil, and C-terminal helix domains. The synthesis of (p)ppGpp and DksA may be altered in response to the stress conditions [32,43]. In this study, A. baumannii ATCC 17978 strain expressed more dksA in harsh culture conditions, such as static conditions and low culture temperature, than in shaking conditions and optimal culture temperature. Mason et al. [32] also demonstrated that the expression of dksA was regulated by different environmental conditions, such as growth phase, growth temperature, and pH in Borrelia burgdorferi. These results suggest that A1S_0248 in A. baumannii ATCC 17978 encodes a DksA.

Herein, we demonstrated that ΔdksA mutants of A. baumannii ATCC 17978 exhibited a filamentous morphology during the specific growth phases (OD600 of 0.95–1.45). Moreover, this mutant strain exhibited growth retardation under stationary conditions but not under shaking conditions. In a previous study, (p) ppGpp-deficient mutants also exhibited more filamentous morphology than the WT A. baumannii ATCC 17978 [37]. The (p)ppGpp-deficient mutants of A. baumannii ATCC 17978 did not show any growth retardation under shaking conditions. However, the growth of (p)ppGpp-deficient mutants under static conditions had not been determined. In E. coli, (p) ppGpp-deficient and ΔdksA mutants exhibit more filamentous morphology than the WT strain [27]. (p) ppGpp and DksA work together to modulate bacterial growth and viability [30,44]. Although the exact mechanisms explaining the differences in bacterial morphology and growth between WT and ΔdksA mutant strains of A. baumannii were not determined in this study, (p)ppGpp and DksA may regulate genes.
associated with cell division or morphogenesis in the same manner.

This study demonstrated that DksA plays an opposite role in biofilm formation and surface motility of A. baumannii ATCC 17978. ΔdksA mutant strains produced a higher biofilm mass than the WT strain, whereas ΔdksA mutants were less motile than the WT strain. The role of (p)ppGpp in biofilm formation was different among bacterial species: (p)ppGpp-deficient mutants of E. coli and Pseudomonas putida formed increased biofilms in LB medium [45,46], whereas (p)ppGpp-deficient mutants of Enterococcus faecalis and Vibrio cholerae formed decreased biofilms [47,48]. As observed in the growth kinetics of A. baumannii strains under static conditions, the ΔdksA mutant exhibited growth retardation at all three tested times in the biofilm assay conditions. The high ability to produce biofilm mass (OD_{570}/OD_{600}) of ΔdksA mutants at 10 h of incubation was due to the growth retardation of this mutant strain. However, biofilm mass (OD_{570}) of the ΔdksA mutant was significantly increased at 24 and 48 h compared with the WT strain, although the growth (OD_{600}) of the ΔdksA mutant was decreased at these times. Genes associated with biofilm formation, such as bfmR/S and csu operon, were upregulated in the ΔdksA mutants, whereas ompA was downregulated in the ΔdksA mutants. In contrast to biofilm formation, the WT strain exhibited more motility than the ΔdksA mutant. The (p)ppGpp-deficient and ΔdksA mutant strains were less motile than the WT E. coli strain [27]. However, the ΔrelA mutant strains of A. baumannii showed hypermotility in motility agar plates [36]. These results suggest that (p)ppGpp and DksA play an opposite role in the surface motility of A. baumannii. Genetic analysis of A. baumannii identified 30 genes that were associated with surface motility, including genes encoding the 1,3-diaminopropane biosynthesis, alarmone/stress metabolism, DNA modification/repair/uptake, lipopeptide synthesis/export, outer membrane proteins, oxidative stress, purine/pyrimidine/folate biosynthesis, RNA modification/regulation, and others [49]. Of the genes associated with surface motility, ompA was downregulated in the ΔdksA mutants. However, the expression of ddc and dat for 1,3-diaminopropane biosynthesis was upregulated in the ΔdksA mutants (Supplementary Figure S2). Altogether, these results suggest that DksA negatively regulates the expression of bfmR/S and csu operon, which modulates the biofilm formation in A. baumannii. However, regulation of genes associated with surface motility of A. baumannii by DksA should be further investigated.

In A. baumannii, 3-hydroxy-dodecanoyl-1-homoserine lactone is a major quorum sensing
molecule [50]. The autoinducers produced by the LuxI-type synthase AbaI bind to their corresponding transcriptional activator AbaR. These complex bind to lux boxes and then activates the transcription of target genes. AbaR is required for expressing abal and producing autoinducers [39]. This quorum sensing system regulates surface motility, biofilm formation, and pellicle formation in A. baumannii [39,50,51]. In P. aeruginosa, (p)ppGpp and DksA negatively regulate the transcription of autoinducer synthase genes, biosynthesis of autoinducers, and inhibit quorum-sensing-dependent virulence factors [52,53]. However, this study showed that dksA is essential for the full expression of abaI and abaR and production of autoinducers in A. baumannii since the ΔdksA mutant exhibited reduced expression of abaI and abaR, and reduced production of autoinducers. Surface motility correlated with the production of autoinducers in ΔdksA mutants, whereas biofilm formation of ΔdksA mutant was increased compared with the WT strain. Our results suggest that DksA regulates the production of autoinducers through the expression of abaI and abaR. However, we cannot exclude the possibility that low production of autoinducers in the ΔdksA mutant results from growth retardation of this mutant strain in bioassay plates because the production of autoinducers depends on cell population density.

The roles of DksA in the pathogenesis of A. baumannii were assessed in an in vivo infection model. In this study, we used neutropenic mice because A. baumannii commonly infects severely ill patients or immunocompromised hosts [1]. CFUs in the lungs of surviving mice were significantly different between the mice infected with WT and ΔdksA mutant strains. Mice survival was also significantly different between WT and ΔdksA mutant strains. However, CFUs in the blood were not different between the two groups because of the big variation in CFUs in each experimental group and the low number of surviving mice. The ΔdksA mutant exhibited lesser virulence than the WT strain in the in vivo mouse model. The ΔdksA mutant exhibited growth retardation under static conditions, filamentous morphology, low expression of ompA, and low production of autoinducers in vitro, possibly contributing to low virulence of ΔdksA mutant in the in vivo infection model. Thus, DksA is a potential target for antivirulence agents against A. baumannii.

This study identifies dksA in A. baumannii. dksA is required to maintain cellular morphology and growth under static conditions in A. baumannii. Furthermore, DksA is directly or indirectly involved in regulating genes associated with virulence factors, which contributes to the pathogenesis of A. baumannii. Overall, A. baumannii DksA plays multifaceted roles in bacterial physiology and virulence. Further studies are required to understand the regulatory mechanisms of multiple genes linked with virulence factors by DksA in A. baumannii.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This work was supported by grants from the Korea Centers for Disease Control and Prevention, Ministry of Health and Welfare, Republic of Korea (2019-ER5401-02).

**Data availability statement**

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

**Author contributions**

NK, MS, and JCL contributed to the design of this study. NK, MS, and JCL contributed to the writing of the manuscript. NK, JHS, and YJK performed the experiments in vitro. NK, KK, and YJK performed the experiments in vivo. NK, MS, and JCL analyzed the data obtained from in vitro and in vivo.

**ORCID**

Minsang Shin http://orcid.org/0000-0001-8679-446X  
Je Chul Lee http://orcid.org/0000-0001-6203-604X

**References**

[1] Antunes LCS, Visca P, Towner KJ. Acinetobacter baumannii: evolution of a global pathogen. Pathog Dis. 2014;71:292–301.

[2] Maragakis LL, Perl TM. Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options. Clin Infect Dis. 2008;46:1254–1263.

[3] Lee CR, Lee JH, Park M, et al. Biology of Acinetobacter baumannii: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options. Front Cell Infect Microbiol. 2017;7:55.

[4] Isler B, Doi Y, Bonomo RA, et al. New treatment options against carbapenem-resistant Acinetobacter baumannii infections. Antimicrob Agents Chemother. 2018;63:e01110–18.

[5] Tacconelli E, Carrara E, Savoldi A, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18:318–327.
[6] Geisinger E, Mortman NJ, Vargas-Cuevas G, et al. A global regulatory system links virulence and antibiotic resistance to envelope homeostasis in Acinetobacter baumannii. PLoS Pathog. 2018;14:e1007030.

[7] Choi CH, Lee EY, Lee YC, et al. Outer membrane protein 38 of Acinetobacter baumannii localizes to the mitochondria and induces apoptosis of epithelial cells. Cell Microbiol. 2005;7:1127–1138.

[8] Gaddy JA, Tomaras AP, Actis LA. The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect Immun. 2009;77:3150–3160.

[9] Smani Y, Dominguez-Herrera J, Pachon J. Association of the outer membrane protein Omp33 with fitness and virulence of Acinetobacter baumannii. J Infect Dis. 2013;208:1561–1570.

[10] Stahl J, Bergmann H, Gottig S, et al. Acinetobacter baumannii virulence is mediated by the concerted action of three phospholipases D. PLoS One. 2015;10:e0138360.

[11] Luke NR, Sauberan SL, Russo TA, et al. Identification and characterization of a glycosyltransferase involved in Acinetobacter baumannii lipopolysaccharide core biosynthesis. Infect Immun. 2010;78:2017–2023.

[12] Weber BS, Harding CM, Feldman MF. Pathogenic Acinetobacter: from the cell surface to infinity and beyond. J Bacteriol. 2015;198:880–887.

[13] Lees-Miller RG, Iwashkiw JA, Scott NE, et al. A common pathway for O-linked protein-glycosylation and synthesis of capsule in Acinetobacter baumannii. Mol Microbiol. 2013;89:816–830.

[14] Gaddy JA, Arivett BA, McConnell MJ, et al. Role of acinetobactin-mediated iron acquisition functions in the interaction of Acinetobacter baumannii strain ATCC 19606 with human lung epithelial cells, Galleria mellonella caterpillars, and mice. Infect Immun. 2012;80:1015–1024.

[15] Nairn BL, Lonergan ZR, Wang J, et al. The response of Acinetobacter baumannii to zinc starvation. Cell Host Microbe. 2016;19:826–836.

[16] Gebhardt MJ, Shuman HA, O’Toole G. GigA and GigB are master regulators of antibiotic resistance, stress responses, and virulence in Acinetobacter baumannii. J Bacteriol. 2017;199:e00066–17.

[17] Eveillard M, Soltner C, Kempf M, et al. The virulence variability of different Acinetobacter baumannii strains in experimental pneumonia. J Infect. 2010;60:154–161.

[18] Irving SE, Corrigan RM. Triggering the stringent response: signals responsible for activating (p)ppGpp synthesis in bacteria. Microbiology. 2018;164:268–276.

[19] Brown A, Fernández IS, Gordyenko Y, et al. Ribosome-dependent activation of stringent control. Nature. 2016;534:277–280.

[20] Zhang Y, Zborníková E, Rejman D, et al. Novel (p)ppGpp binding and metabolizing proteins of Escherichia coli. mBio. 2018;9:e02188–17.

[21] Roberts JW. Promoter-specific control of E. coli RNA polymerase by ppGpp and a general transcription factor. Genes Dev. 2009;23:143–146.

[22] Gourse RL, Chen AY, Gopalkrishnan S, et al. Transcriptional responses to ppGpp and DksA. Annu Rev Microbiol. 2018;72:163–184.

[23] Ross W, Sanchez-Vazquez P, Chen AY, et al. ppGpp binding to a site at the RNAP-DksA interface accounts for its dramatic effects on transcription initiation during the stringent response. Mol Cell. 2016;62:811–823.

[24] Paul BJ, Barker MM, Ross W, et al. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell. 2004;118:311–322.

[25] Parshin A, Shiver AL, Lee J, et al. DksA regulates RNA polymerase in Escherichia coli through a network of interactions in the secondary channel that includes Sequence Insertion 1. Proc Natl Acad Sci U S A. 2015;112:E6862–E6871.

[26] Paul BJ, Berkmen MB, Gourse RL. DksA potentiates direct activation of amino acid promoters by ppGpp. Proc Natl Acad Sci U S A. 2005;102:7823–7828.

[27] Magnusson LU, Gummesson B, Joksimović P, et al. Identical, independent, and opposing roles of ppGpp and DksA in Escherichia coli. J Bacteriol. 2007;189:5193–5202.

[28] Tehranchi AK, Blankschien MD, Zhang Y, et al. The transcription factor DksA prevents conflicts between DNA replication and transcription machinery. Cell. 2010;141:595–605.

[29] Myka KK, Kusters K, Washburn R, et al. DksA-RNA polymerase interactions support new origin formation and DNA repair in Escherichia coli. Mol Microbiol. 2019;111:1382–1397.

[30] Ishii Y, Yamada H, Yamashino T, et al. Deletion of the yhhP gene results in filamentous cell morphology in Escherichia coli. Biosci Biotechnol Biochem. 2000;64:799–807.

[31] Henard CA, Bourret TJ, Song M, et al. Control of redox balance by the stringent response regulatory protein promotes antioxidant defenses of Salmonella. J Biol Chem. 2010;285:36785–36793.

[32] Mason C, Thompson C, Ouyang Z. DksA plays an essential role in regulating the virulence of Borrelia burgdorferi. Mol Microbiol. 2020;114:172–183.

[33] Sharma AK, Payne SM. Induction of expression of hfq by DksA is essential for Shigella flexneri virulence. Mol Microbiol. 2006;62:469–479.

[34] Wang J, Cao L, Yang X, et al. Transcriptional analysis reveals the critical role of RNA polymerase-binding transcription factor, DksA, in regulating multi-drug resistance of Escherichia coli. Int J Antimicrob Agents. 2018;52:63–69.

[35] Das B, Bhadra RK. (p)ppGpp metabolism and antimicrobial resistance in bacterial pathogens. Front Microbiol. 2020;11:563944.

[36] Pérez-Varela M, Tierney ARP, Kim JS, et al. Characterization of RelA in Acinetobacter baumannii. J Bacteriol. 2020;202:e00045–20.
[37] Jung HW, Kim K, Islam MM, et al. Role of ppGpp-regulated efflux genes in Acinetobacter baumannii. J Antimicrob Chemother. 2020;75:1130–1134.

[38] Oh MH, Lee JC, Kim J, et al. Simple method for markerless gene deletion in multidrug-resistant Acinetobacter baumannii. Appl Environ Microbiol. 2015;81:3357–3368.

[39] Oh MH, Han K. AbaR is a LuxR type regulator essential for motility and the formation of biofilm and pellicle in Acinetobacter baumannii. Genes Genomics. 2020;42:1339–1346.

[40] Zhang L, Murphy PJ, Kerr A, et al. Agrobacterium conjugation and gene regulation by N-acyl-L-homoserine lactones. Nature. 1993;362:446–448.

[41] Park SY, Lee SJ, Oh TK, et al. AhlD, an N-acylhomoserine lactonase in Arthrobacter sp., and predicted homologues in other bacteria. Microbiology. 2003;149:1541–1550.

[42] Na SH, Oh MH, Jeon H, et al. Imaging of bioluminescent Acinetobacter baumannii in a mouse pneumonia model. Microbiol Pathog. 2019;137:103784.

[43] Dalebroux ZD, Svensson SL, Gaynor EC, et al. ppGpp conjugates bacterial virulence. Microbiol Mol Biol Rev. 2010;74:171–199.

[44] Kriel A, Bittner AN, Kim SH, et al. Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. Mol Cell. 2012;48:231–241.

[45] Balzer GJ, McLean RJC. The stringent response genes relA and spoT are important for Escherichia coli biofilms under slow-growth conditions. Can J Microbiol. 2002;48:675–680.

[46] Liu H, Xiao Y, Nie H, et al. Influence of (p)ppGpp on biofilm regulation in Pseudomonas putida KT2440. Microbiol Res. 2017;204:1–8.

[47] Chavez De Paz LE, Lemos JA, Wickstrom C, et al. Role of (p)ppGpp in biofilm formation by Enterococcus faecalis. Appl Environ Microbiol. 2012;78:1627–1630.

[48] He H, Cooper JN, Mishra A, et al. Stringent response regulation of biofilm formation in Vibrio cholerae. J Bacteriol. 2012;194:2962–2972.

[49] Blaschke U, Skiebe E, Wilharm G. Novel genes required for surface-associated motility in Acinetobacter baumannii. Curr Microbiol. 2021;78:1509–1528.

[50] Niu C, Clemmer KM, Bonomo RA, et al. Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii. J Bacteriol. 2008;190:3386–3392.

[51] Stacy DM, Welsh MA, Rather PN, et al. Attenuation of quorum sensing in the pathogen Acinetobacter baumannii using non-native N-acyl homoserine lactones. ACS Chem Biol. 2012;7:1719–1728.

[52] Branny P, Pearson JP, Pesci EC, et al. Inhibition of quorum sensing by a Pseudomonas aeruginosa dksA homologue. J Bacteriol. 2001;183:1531–1539.

[53] Schalhauser J, Lepine F, McKay G, et al. The stringent response modulates 4-hydroxy-2-alkylquinoline biosynthesis and quorum-sensing hierarchy in Pseudomonas aeruginosa. J Bacteriol. 2014;196:1641–1650.