Short Communication

# Effects of prophage regions in a plasmid carrying a carbapenemase gene on survival against antibiotic stress

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## ARTICLE INFO

## ABSTRACT

We investigated the evolutionary importance of cryptic prophage elements in a $bla_{NDM-1}$-bearing plasmid by exploring the effect of prophage regions on survival against antibiotic stress. While analysing a plasmid harboring an NDM-1-encoding gene in *Klebsiella pneumoniae* from South Korea, we found a prophage region within the plasmid. We constructed single-prophage knockout (KO) mutants by gene replacement. The intact plasmid and plasmids with deleted prophages were conjugated into *Escherichia coli* DH5$\alpha$. Growth rate and antibiotic susceptibility were determined, and survival rates of strains were evaluated in the presence of antibiotics, such as imipenem, amikacin, gentamicin, cefotaxime, and piperacillin/tazobactam. A transcriptional response of sigma factor-coding genes (*rpoS* and *rpoE*) and reactive oxygen species (ROS)-related genes from different operons (*soxS, fumC, oxyR,* and *katE*) to a subinhibitory concentration of aminoglycosides was monitored by quantitative real-time polymerase chain reaction (qRT-PCR). The prophage region consists of four cryptic prophages of 16,795 bp and 19 coding DNA sequences. An *Escherichia coli* transconjugant carrying the plasmid with intact prophages showed increased survival during treatment with various antibiotics, including imipenem and amikacin; however, transconjugants carrying this plasmid with a single-prophage KO did not. mRNA expression analyses revealed that sigma factor proteins (rpoB and rpoE) were highly upregulated by antibiotics. We propose that cryptic prophages in the antibiotic resistance plasmid may contribute to adaptation of the bacterial host to antibiotic stress. We are concerned that the combination of prophages with a drug resistance plasmid helps drug-resistant bacteria in a hostile environment and accelerates their dissemination.

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## 1. Introduction

Carbapenem resistance in Gram-negative bacteria is primarily mediated by the production of carbapenemases hydrolyzing the $\beta$-lactam ring of carbapenem antibiotics. Since the first identification in *Klebsiella pneumoniae*, the $bla_{NDM-1}$ gene has been detected in a wide range of bacterial species in many countries worldwide [1,2]. Generally, $bla_{NDM}$ is carried by a plasmid and is horizontally transferred within and between species, contributing to the rapid dissemination of NDM-producing pathogens [3].

The cryptic prophages trapped in the chromosome, which constitutes up to 25% of bacterial chromosomal DNA, are important for the evolution of bacterial hosts with respect to virulence and fitness [4,5]. Beneficial effects of prophages on bacterial physiology include the ability to grow more rapidly and to obtain higher yields on nutrients, and they are also involved in a general stress response and increased resistance to antibiotics [5–7]. Thus, phages embedded in a bacterial chromosome are believed to significantly increase bacterial fitness [4-6].

Several antibiotic resistance plasmids bearing prophage(s) have been reported [8-10]. Although biological roles of cryptic prophages located on a bacterial chromosome have been extensively studied, the functions of prophages incorporated into a plasmid have rarely been explored [5,6]. In the present study, we show that cryptic prophages may protect the host bacterium from antibiotic stress. In addition, prophages participate in bacterial fitness by regulating sigma factors, which activate general stress responses that protect the bacterial cell from antibiotics.

## 2. Materials and Methods

### 2.1. The bacterial strain

The bacterial strain, A1, used in this study is one of three NDM-1-resistant *K. pneumoniae* ST340 isolates that were obtained at a single tertiary care hospital in South Korea in October 2011 [11].

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## 2.2. Plasmid sequencing and bioinformatic analysis

A $bla_{NDM-1}$-carrying plasmid (pNDM-A1) was extracted by the conjugation method using the *K. pneumoniae* strain as a donor and streptomycin-resistant *E. coli* strain DH5$\alpha$ as a recipient. Complete sequencing of the $bla_{NDM-1}$-carrying plasmid was performed using a Roche 454 GS-FLX system (v. 2.9). A total of 24 978 sequence reads were generated, yielding a mean sequence coverage of $186 \times$ for plasmid pNDM-A1. The PHAge Search Tool (PHAST) was used on the nucleic acid level for identification of integrated bacteriophages in the chromosomes [12]. The annotated sequences of plasmid pNDM-A1 were submitted to the GenBank nucleotide sequence database (GenBank accession numbers MF511773).

## 2.3. Construction of single-prophage knockout (KO) mutants by gene replacement

The strains and plasmids used in this study are listed in Supplementary Table S1. To delete four prophage regions individually in plasmid DH5$\alpha$/pNDM-A1, we expanded the use of the one-step inactivation method that was originally devised for deletion of single-prophage regions [13]. After one-step inactivation using primers flanking the prophage (Supplementary Table S2), the four prophage regions were replaced with a kanamycin resistance cassette (~1.5 kb) from plasmid pKD4. The two prophages rac (7,118 bp) and e14 (2,722 bp) and KpLE2 phage-like elements—*yjhFG* (3,454 bp) and *yjhQXZR* (2,793 bp)—were individually removed from the NDM-1-carrying plasmid in *E. coli* DH5$\alpha$.

## 2.4. Determination of the growth rate

Growth rates were determined for isogenic strains *E. coli* DH5$\alpha$, DH5$\alpha$ with intact pNDM-A1, and DH5$\alpha$ transconjugants carrying a plasmid with a single-prophage deletion (pA1$\triangle$rac, pA1$\triangle$e14, pA1$\triangle$KpLE2-1, and pA1$\triangle$KpLE2-2). Growth rates were determined by incubating bacteria in Luria-Bertani (LB) broth at 37°C for 24 h and 8 h for a maximal specific growth rate. Cells from overnight LB cultures were transferred to fresh LB broth at initial $OD_{600}$ of 0.05, and the growth was quantified every hour for 10 hours by means of $OD_{600}$.

## 2.5. Determination of antibiotic susceptibility

To determine minimum inhibitory concentrations (MICs) of the 17 antibiotics, in vitro antibiotic susceptibility testing was conducted by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. Susceptibility was defined according to CLSI breakpoints, and *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 served as control strains.

The survival rates of strains were evaluated in the presence of antibiotics (imipenem, amikacin, gentamicin, cefotaxime, and piperacillin/tazobactam). Overnight cultures of the strains were reinoculated to attain the concentration of $10^6$ colony-forming units (cfu)/mL in Mueller-Hinton (MH) broth at various concentrations of antibiotics based on MIC values and were incubated at 37°C for 18 h. The cultures were diluted and plated onto drug-free MH agar for colony counting.

## 2.6. Evaluation of relative gene expression during an antibiotic stress response

To investigate the regulation of stress-related genes (*rpoS, rpoE, soxS, fumC, oxyR*, and *katE*) under antibiotic stress, overnight cultures were set up to attain turbidity of 0.5 and then were exposed to antibiotics, 2 mg/L amikacin and 0.25 mg/L gentamicin. The antibiotics were added to the culture medium and incubated for 18 h. Relative amounts of mRNA transcripts of stress-related genes in *E. coli* under diverse conditions were determined by quantitative real-time PCR (qRT-PCR) (Supplementary Table S3). Housekeeping gene *rpoB* was used to normalize gene expression by the threshold cycle ($C_T$) method. The quantification method based on the relative amount of a target gene vs. a reference gene was used.

## 2.7. Statistical analysis

Statistical analyses were performed as appropriate by Student's *t* test or ANOVA, followed by the Dunnett multiple-comparison test in Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

# 3. Results

## 3.1. Complete sequence of plasmid pNDM-A1

The $bla_{NDM-1}$-bearing pNDM-A1 plasmid was found to be 67,644 bp in size (Supplementary Fig. S1). It contains 67 predicted open reading frames (ORFs). Replicon typing revealed that the plasmid belongs to an IncX subgroup, IncX3. BLASTn search in the NCBI database revealed that pNDM-A1 shares high homology with previously characterized plasmids: pNDM-ECN49 of *Enterobacter cloacae* isolated in China in 2015 (GenBank accession No. KP765744) and pIncX-SHV of *K. pneumoniae* discovered in Italy in 2012 (GenBank accession No. NC_019157). Although $bla_{SHV-11}$, an extended spectrum $\beta$-lactamase gene, was identified in pIncX-SHV, pNDM-ECN49 carries $bla_{NDM-1}$ as does pNDM-A1.

## 3.2. Features of the prophage region

The prophage region was identified in the pNDM-A1 plasmid and is 16,795 bp in size, and comprises four different cryptic prophages (rac, e14, KpLE2-1, and KpLE2-2) including 19 ORFs (Supplementary Fig. S1). The genes within the prophage region are intact and are similar to those in prophages found on the *E. coli* chromosome: BLASTn comparison revealed that rac prophage genes, e14 prophage genes, and KpLE2 phage-like elements share 99% nucleotide identity and 100% coverage with those of *E. coli* K12 strain MG1655 (NZ_CP009685). Genes known to be essential for phage activities, such as DNA transcriptional processing (*ydaS, ydaV, ydaW, ynaK*, and *ydaY*), transporter subunits (*trkG* and *yjhF*), and phage-structural components (*tmpR*), were identified.

## 3.3. The growth rate

We compared growth rates of a wild-type (WT) recipient strain (*E. coli* DH5$\alpha$) and five DH5$\alpha$ transconjugants carrying intact pNDM-A1 or this plasmid with deletion of a single prophage region: strains DH5$\alpha$/pNDM-A1, DH5$\alpha$/pA1$\triangle$rac, DH5$\alpha$/pA1$\triangle$e14, DH5$\alpha$/pA1$\triangle$KpLE2-1, and DH5$\alpha$/pA1$\triangle$KpLE2-2 (Fig. 1A). The transconjugant with the intact plasmid showed no difference in growth rate compared with the WT DH5$\alpha$ strain, which implies that the plasmid with the antibiotic resistance gene and cryptic prophages represents no burden to the bacterial host for growth. In contrast, transconjugants carrying a plasmid with a single-prophage KO grew more slowly for 8 h compared with WT strain DH5$\alpha$ and strain DH5$\alpha$/pNDM-A1, although they grew at a rate similar to that of the WT *E. coli* strain and strain DH5$\alpha$/pNDM-Al in the stationary phase.
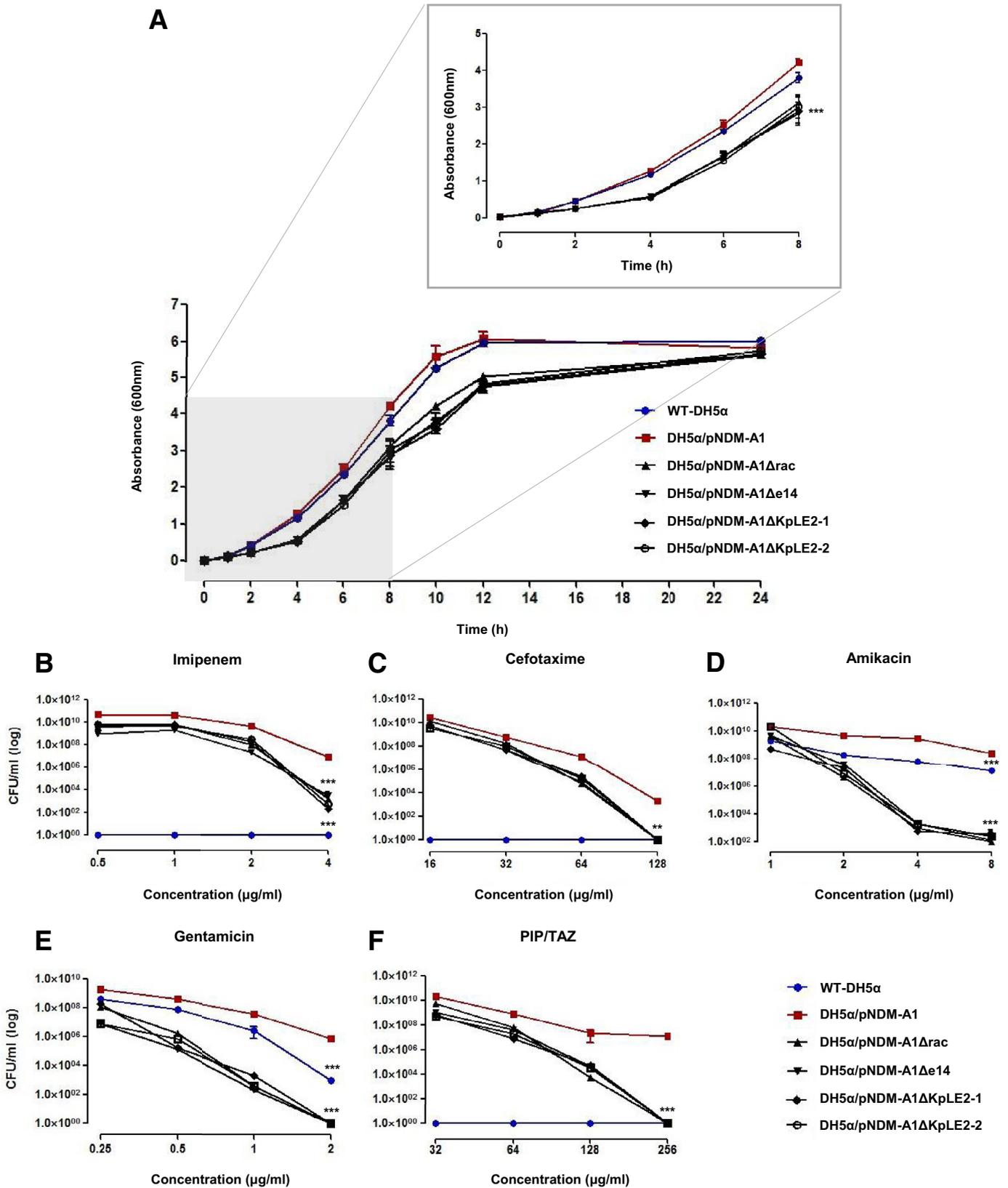
**Fig. 1.** The pNDM-A1 plasmid bearing intact prophages increases cell growth and resistance to antibiotics. Bacterial growth curves of transconjugants carrying either the intact plasmid or the plasmid with a single-prophage KO in assays lasting for 8 h and for 24 h (A). The growth rates in LB broth at 37°C were monitored by absorbance at OD600 in WT *E. coli* DH5α, DH5α/pNDM-A1, and four transconjugants carrying the plasmid lacking a prophage gene. Growth retardation in the exponential growth phase was observed in transconjugants carrying the plasmid with a single-prophage KO. Error bars indicate SD values (n = 4). The survival rates were evaluated for 20 h for each of the four drug concentrations in Mueller-Hinton (MH) agar containing one of five antibiotics: (B) imipenem, (C) cefotaxime, (D) amikacin, (E) gentamicin, or (F) piperacillin-tazobactam. The transconjugant with the intact plasmid (DH5α/pNDM-A1) showed a higher survival rate in the presence of each antibiotic tested in this study compared with WT DH5α, and survival rates of transconjugants carrying the plasmid with a single-prophage KO decreased compared with DH5α/pNDM-A1. Statistical significance was evaluated by comparison with a transconjugant carrying the intact plasmid (DH5α/pNDM-A1); *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

**Table 1**
MICs of the pNDM-A1-carrying transconjugant and its single-prophage knockout (KO) mutants.

| Antimicrobial agents[a] | MIC (mg/L)[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Donor | Recipient | Transconjugant with intact plasmid | Transconjugants with single-prophage KO in plasmid | | | |
| | A1 | DH5α | DH5α/pNDM-A1 | DH5α/pA1△rac | DH5α/pA1△e14 | DH5α/pA1△KpLE2-1 | DH5α/pA1△KpLE2-2 |
| **Carbapenems** | | | | | | | |
| Imipenem | 8 | 0.25 | **4** | **2\*** | **2\*** | **2\*** | **2\*** |
| Meropenem | 16 | 0.06 | **2** | **1\*** | **1\*** | **1\*** | **1\*** |
| **Polymyxins** | | | | | | | |
| Colistin | 4 | 0.25 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Polymyxin B | 4 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| **Cephalosporins** | | | | | | | |
| Cefotaxime | >128 | 0.125 | **128** | **64\*** | **64\*** | **64\*** | **64\*** |
| Ceftazidime | >64 | 0.5 | **>64** | >64 | >64 | >64 | >64 |
| Cefepime | >64 | 1 | **>64** | **16\*** | **16\*** | **16\*** | **16\*** |
| Ceftriaxone | >128 | 0.25 | **>128** | **64\*** | **128\*** | **128\*** | **128\*** |
| **Penicillins** | | | | | | | |
| Ampicillin | >64 | 4 | **>64** | >64 | >64 | >64 | >64 |
| **Aminoglycosides** | | | | | | | |
| Gentamicin | 4 | 4 | **8** | **1\*** | **1\*** | **1\*** | **1\*** |
| Amikacin | 32 | 32 | 32 | **4\*** | **4\*** | **4\*** | **4\*** |
| **Monobactam** | | | | | | | |
| Aztreonam | >64 | 2 | **>64** | >64 | >64 | >64 | >64 |
| **Fluoroquinolones** | | | | | | | |
| Ciprofloxacin | >64 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| **Tetracyclines** | | | | | | | |
| Tetracycline | >64 | 1 | 1 | **0.5\*** | **0.5\*** | **0.5\*** | **0.5\*** |
| Tigecycline | 1 | 0.125 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| **Others** | | | | | | | |
| SXT | 0.5/9.5 | 0.125/2.375 | 0.125/2.375 | 0.125/2.375 | 0.125/2.375 | 0.125/2.375 | 0.125/2.375 |
| P/T | >256/4 | 2/4 | **>256/4** | **128/4\*** | **128/4\*** | **128/4\*** | **128/4\*** |

[a] SXT, trimethoprim/sulfamethoxazole; P/T, piperacillin/tazobactam.

[b] MIC measurements were repeated five times, and the median MIC values are presented. MICs that are greater in the transconjugant than in the recipient (*E. coli* DH5α) are shown in boldface. Boldfaced and asterisked MICs indicate that the bacteria were ≥2-fold less resistant than strain DH5α/pNDM-A1 (pNDM-A1 carrying four prophage regions in *E. coli*).

## 3.4. Antibiotic resistance

Compared with WT *E. coli* strain DH5α, the transconjugant with the intact plasmid (DH5α/pNDM-A1) showed higher MICs for carbapenems, cephalosporins, aminoglycosides, monobactam, ampicillin, and piperacillin/tazobactam (Table 1). Deletion of a prophage in the plasmid affected antibiotic susceptibility of the bacterial host. The carbapenem MICs in the four transconjugants carrying the plasmid with a single-prophage KO decreased twofold compared with strain DH5α/pNDM-Al carrying the intact plasmid (Table 1). Reduced MICs in the transconjugants carrying the plasmid with a single-prophage KO were also seen for cephalosporins, aminoglycosides, tetracycline, and piperacillin/tazobactam.

The increase in antibiotic resistance due to the plasmid with $bla_{NDM-1}$ and the decrease in antibiotic resistance because of the deletion of a prophage in the plasmid were also revealed in experiments on bacterial cell viability at different antibiotic concentrations (Figs. 1B to 1F). During treatment with one of five antibiotics (imipenem, cefotaxime, amikacin, gentamicin, and piperacillin/tazobactam), strain DH5α/pNDM-A1 showed significantly higher survival rates than WT *E. coli* DH5α. In addition, survival in the presence of those antibiotics decreased significantly in the transconjugants carrying the plasmid with a single-prophage KO.

## 3.5. A transcriptional response to a sub-inhibitory concentration of aminoglycosides

We evaluated the transcriptional changes of sigma factor-coding genes (*rpoS* and *rpoE*) and reactive oxygen species (ROS)-related genes from different operons (*soxS, fumC, oxyR*, and *katE*) in *E. coli* transconjugants when sub-inhibitory concentrations of amikacin

and gentamicin were added to the culture medium (Figs. 2A and 2B). Although *rpoS* and *rpoE* were not activated in strain DH5α/pNDM-A1 compared with WT DH5α during treatment with either aminoglycoside antibiotic, they were repressed significantly in transconjugants carrying the plasmid with a single-prophage KO in most cases (Figs. 2A and 2B and Supplementary Table S4). On the other hand, ROS-related genes *soxS, oxyR*, and *katE* were overexpressed in strain DH5α/pNDM-A1 and were downregulated in transconjugants carrying the plasmid with a single-prophage KO during treatment with amikacin (Fig. 2A). Although *fumC* was not overexpressed in DH5α/pNDM-A1, it was repressed in the presence of amikacin in all four types of transconjugants carrying the plasmid with a single-prophage KO. When gentamicin was added to the culture medium, only *soxS* was overexpressed significantly in strain DH5α/pNDM-A1 and was repressed in all the four transconjugants with a single-prophage KO (Fig. 2B). Overexpression of *katE* in strain DH5α/pNDM-A1 during treatment with gentamicin was not significant, but its repression was evident in transconjugants with a single-prophage KO. Transcriptional changes of *fumC* and *oxyR* during treatment with gentamicin were not detected or were not significant except in some cases. This finding indicates that sub-inhibitory concentrations of aminoglycosides increase the cellular amount of antioxidant proteins and sigma factor proteins, which may be affected by prophages of the antibiotic resistance plasmid.

## 4. Discussion

NDM-producing Enterobacteriaceae have been isolated from humans and various environments and have spread worldwide [1]. In this study, we determined the whole sequence of a $bla_{NDM-1}$-carrying plasmid of *K. pneumoniae* strain A1 originating from a
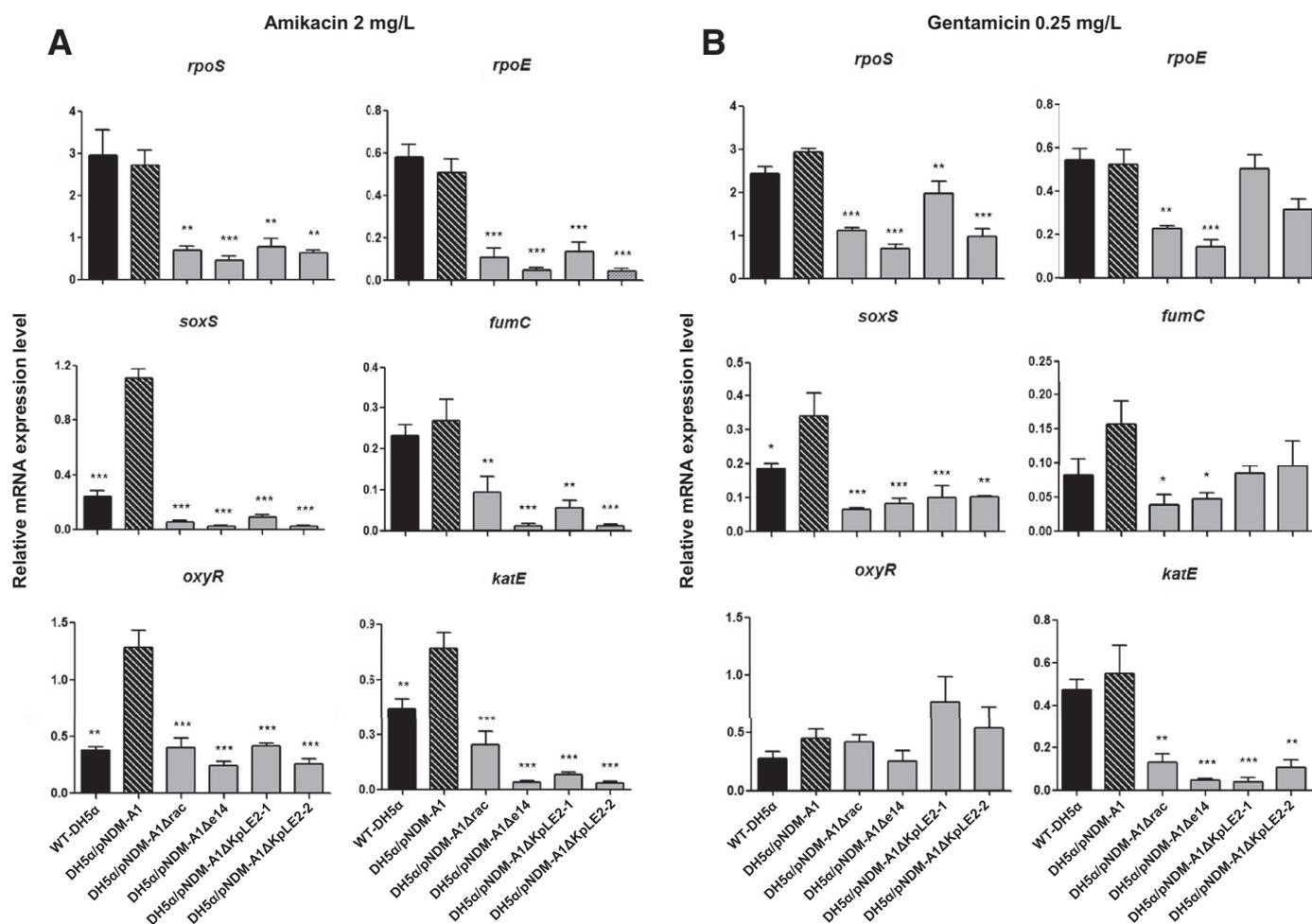
**Fig. 2.** The transcriptional response of genes encoding sigma factors (*rpoS* and *rpoE*) and ROS-related genes (*soxS, fumC, oxyR,* and *katE*) when bacteria were treated with sub-inhibitory concentration of aminoglycoside antibiotics: (A) amikacin (2 mg/L for 18 h) or (B) gentamicin (0.25 mg/L for 18 h). The transcription levels were measured by qRT-PCR. Although the transcription of sigma factors did not differ between WT *E. coli* DH5α and DH5α/pNDM-A1, these transcription levels were significantly decreased in transconjugants carrying the plasmid with a single-prophage KO. Overall, ROS-related genes were overexpressed in strain DH5α/pNDM-A1 and were repressed in the transconjugants carrying the plasmid with a single-prophage KO. Statistical significance was evaluated by comparison with the transconjugant carrying the intact plasmid (DH5α/pNDM-A1); *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

Korean patient [5]. The characteristic feature of the plasmid is the presence of cryptic prophage regions. A comparison of plasmid structures indicates that an approximately 21-kb $bla_{NDM-1}$ genetic module (where the cryptic prophage regions were inserted) recombined with an IncX3-structured plasmid with the help of insertion sequence (IS) elements and the other mobile transposon elements flanking the junctions.

A phage-related element was recently highlighted as a mobile genetic element that transfers antibiotic resistance [8]. In addition, increasing evidence shows that prophages of commensal and environmental bacteria are reservoirs of antibiotic resistance, virulence, and stress tolerance, and their participation in the dissemination of high-risk pathogenic bacteria through horizontal gene transfer should be recognized [15].

In the present study, we showed that a cryptic prophage system of plasmid pNDM-A1 may help to protect the bacterial host from adverse hostile conditions, such as the presence of antibiotics. Of note, the beneficial effects of prophages against antibiotics were evident only if all four prophages of pNDM-A1 were intact; these effects disappeared even if only one of the four prophages of pNDM-A1 was deleted.

The finding that the $bla_{NDM-1}$-bearing plasmid contributes to reduced sensitivity to $\beta$-lactam antibiotics, including carbapenems, in a bacterial transconjugant (DH5α/pNDM-A1) was expected.

Nonetheless, it was not anticipated that the MICs and survival rates in relation to aminoglycosides and monobactams would also be increased because no corresponding resistance genes were found in the plasmid in this study. In addition, the transconjugants carrying the plasmid with a single-prophage KO showed reduced antibiotic resistance despite the presence of an intact carbapenemase gene, $bla_{NDM-1}$. It is noteworthy that the MICs of carbapenems and cephalosporins for the transconjugants carrying the plasmid with a single-prophage KO did not decrease to the level of the susceptible recipient strain. Thus, the cryptic prophage of the plasmid may partially contribute to the antibiotic resistance.

The prophage system may affect universal regulatory systems of bacteria. The sigma factor proteins, such as $\delta^S$ (*rpoS*), $\delta^E$ (*rpoE*), and $\delta^H$ (*rpoH*), were highly upregulated under the influence of antibiotics when the plasmid with intact prophages was transconjugated into the bacterial host. The alternative sigma factors activate a general stress response and protect the bacterial cell from harmful environmental conditions, including antibiotic pressure, via increased transcription of genes belonging to general-stress-related regulons [16]. It is well known that steady induction of *rpoS* and *rpoE* can lead to more efficient protection from oxidative stress and can prevent DNA damage in *E. coli* [17].

The genes overexpressed under stress in strain DH5α/pNDM-A1 were mostly downregulated to the level of the WT strain in

transconjugants carrying the plasmid with a single-prophage KO; this finding agrees with the results of our survival analyses. It implies that all four prophages are necessary for increased survival of the bacterial host or for persistence of the plasmid in a hostile environment. This finding may contradict the results on phages located on a bacterial chromosome because most mutants carrying the plasmid with a single-prophage KO here showed increased cell viability during exposure to stressors, including antibiotics. Although prophages rac and e14 were found to be common in the study by Wang et al. [6], in our study, their deletion yielded different results. Four prophages in our study might have recombined with the antibiotic resistance plasmid *en bloc* and perform their function only if they are all intact.

Antibiotic resistance is a major threat worldwide [18]. Horizontal gene transfer mediated by a plasmid is one of the main causes of increased resistance rates and rapid dissemination of antibiotic-resistant pathogens. Although antibiotic resistance may be a burden deleterious to bacteria compared with a susceptible strain, bacteria frequently overcome such fitness cost by several mechanisms, such as hypermutation, compensatory mutation, and cross-selection [19]. Cryptic prophages in an antibiotic resistance plasmid may contribute to the persistence of the resistant strain in diverse environments (including antibiotic pressure) and to its spread.

In this study, we found cryptic prophages in a plasmid carrying a carbapenem resistance gene and showed that they may provide a survival advantage to the bacterial host under antibiotic pressure by controlling the mRNA expression of universal regulatory systems in bacteria. This notion indicates that prophages in the plasmid may represent a refractory mechanism of antibiotic resistance.

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## Declarations

## Funding

## Competing Interests

None declared.

## Ethical Approval

Not required.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.09.002.

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