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ORIGINAL ARTICLE

DNA adenine methylation modulates pathogenicity of *Klebsiella pneumoniae* genotype K1

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Abstract *Background/Purpose: Klebsiella pneumoniae* genotype K1 is a highly virulent pathogen that causes liver abscess and metastatic endophthalmitis/meningitis. Whether its pathogenicity is controlled by DNA adenine methylase (Dam), an epigenetic regulator of bacterial virulence gene expression, is yet unknown. We aimed to study the role of DNA adenine methylation in the pathogenicity of *K. pneumoniae* genotype K1.
Methods: We identified the *dam* gene in the prototype tissue-invasive strain (NTUH-K2044) of *K. pneumoniae* genotype K1, using the strain's complete genome sequence in GenBank. We constructed a *dam*$^-$ mutant and compared it with the wild type, in terms of *in vitro* serum resistance and *in vivo* BALB/cByl mice inoculation.
Results: Loss of Dam activity in the mutant was verified by *Mbo*I restriction digestion of the genomic DNA and a 1000-fold increase in spontaneous mutation rate. The *dam* mutant lost at least 68% of serum resistance when compared with the wild type (survival ratio at 1 hour: 2.6 ± 0.4 vs. 8.2 ± 1.9; at 2 hours: 3.9 ± 1.6 vs. 17.4 ± 3.6; p values < 0.05). Likewise, virulence to mice decreased by 40-fold in an intraperitoneal injection model [lethal dose, 50% (LD_{50}): 2×10^3 colony-forming units (CFUs) vs. 5×10^1 CFUs] and by sixfold in a gastric ingestion model (LD_{50}: 3×10^4 CFUs vs. 5×10^3 CFUs). Attenuation of the *dam* mutant was not attributable to its growth rate, which was similar to that of the wild type.

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Conclusion: Our results support the view that DNA adenine methylation plays an important role in modulating the pathogenicity of *K. pneumoniae* genotype K1. The incomplete attenuation indicates the existence of other regulatory factors.

Introduction

Klebsiella pneumoniae typically causes pneumonia, urinary tract infection, and other infections in hospitalized patients with compromised host immunity.[1] In contrast to classical *K. pneumoniae*, a hypervirulent type of *K. pneumoniae*—genotype K1—causes a distinctive syndrome of community-acquired pyogenic liver abscess that is complicated by metastatic endophthalmitis or central nervous system (CNS) infections even in healthy persons.[2] Despite aggressive therapy, the outcome frequently involves catastrophic disability such as loss of vision, quadriplegia, paraparesis, and impaired higher cortical functions.[2] This hypervirulent *K. pneumoniae* is characterized by hypermucoviscous colony and a high resistance to human serum that facilitates its spread via bloodstream.[3] The enhanced pathogenicity of *K. pneumoniae* genotype K1 has been attributed to a combination of several virulence factors, including K1 capsular polysaccharides,[2] O1 lipopolysaccharides,[4] large plasmid-encoded *rmpA* and aerobactin,[5,6] as well as other iron-acquisition systems.[7–9] However, little is known about the regulatory mechanisms that control and coordinate the production of the virulence factors to facilitate pathogenesis in this emerging pathogen.

DNA methylation, which alters the affinity and interaction of regulatory proteins with DNA, is an epigenetic mechanism that regulates numerous bacterial physiological processes including chromosome replication, DNA segregation, mismatch repair, transposition, and transcription.[10] DNA adenine methylase (Dam), which methylates the N-6 of adenine in the GATC sequence, plays a key role in bacterial virulence gene expression.[10–12] Using Dam-deficient mutants or Dam-overproducing strains (in cases when Dam is essential for viability), studies have shown that dysregulation in Dam function leads to virulence attenuation in various pathogens, such as *Salmonella typhimurium*,[11] *Haemophilus influenzae*,[13] *Yersinia pestis*,[14] *Yersinia pseudotuberculosis*,[15] *Vibrio cholerae*,[16] *Aeromonas hydrophila*,[17] and *Pasteurella multocida*.[18] However, the effects of *dam* mutations differ among bacteria. Dam is unrelated to virulence in *Neisseria meningitides* and *Shigella flexneri*.[19,20] In non-K1 *K. pneumoniae*, inactivation of Dam causes only partial attenuation, as shown in a K2 strain that was virulent in a murine model of infection.[21] To date, no study has specifically investigated the role of Dam in the pathogenicity of *K. pneumoniae* genotype K1, which has a genomic background distinctively different from that of non-K1 *K. pneumoniae* strains.[22] Filling the knowledge gap is crucial, for example, to assess the potential of Dam-targeted antimicrobial or immunization strategies for preventing the devastating outcomes associated with this highly virulent pathogen.[2]

We aimed to study the role of DNA adenine methylation in the pathogenicity of *K. pneumoniae* genotype K1, using the prototype tissue-invasive strain NTUH-K2044.[2,3] We constructed a *dam*$^-$ mutant and confirmed its loss of Dam activity. We then compared the mutant with the wild type on pathogenicity, using *in vitro* serum resistance assays and *in vivo* BALB/cByl mice inoculation tests, which serve as surrogate markers for the pathogenicity of hypervirulent *K. pneumoniae* in humans.[2,3,7]

Methods

Ethical statement

The animal research protocol was reviewed and approved by the Institutional Animal Care and Use Committee of National Taiwan University College of Medicine and College of Public Health (Taipei, Taiwan).

Bacterial strains, plasmids, and growth conditions

NTUH-K2044, the prototype strain of *K. pneumoniae* genotype K1, caused community-acquired pyogenic liver abscess with metastatic endophthalmitis and meningitis in a previously healthy 40-year-old man.[3] Table 1 lists the strains and plasmids used in this study.[23–25] All strains were stored at −80°C before use. For general use, both *K. pneumoniae* and *Escherichia coli* were grown in the Luria—Bertani (LB) broth or agar at 37°C. For the *dam* mutant, kanamycin 50 μg/mL was added to the LB medium.

Cloning of the *dam* gene in *K. pneumoniae*

We searched the complete genome sequence of *K. pneumoniae* strain NTUH-K2044 (GenBank accession numbers AP006725 and AP006726) to identify open reading frames (ORFs) matching the published amino acid sequence of Dam from *E. coli* K-12 (GenBank accession number AAC76412). The website http://genome.nhri.org.tw/KP/ offers built-in Basic Local Alignment Search Tool (BLAST) search tools. We amplified the *dam* gene from the extracted genomic DNAs of *K. pneumoniae* NTUH-K2044 and MGH-78578 for cloning and DNA sequencing.

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
Klebsiella pneumoniae strains		
NTUH-K2044	Hypervirulent *K. pneumoniae* strain (K1)	NTUH
MGH-78578	Classical *K. pneumoniae* strain (K52)	ATCC
NTUH-K5906	Serum-sensitive strain, used as control in serum resistance assay	This work
Escherichia coli strains		
RS5033	*dam*$^-$ Strain	Fang et al[23]
S17-1/*λpir*	Donor of pUTKm1Δ*tnp*Δ*bla*Δ*mini-Tn5* during conjugation	de Lorenzo et al[24]
Plasmids		
pGEM T-Easy	Vector for TA cloning	Promega
pUTKm1Δ*tnp* Δ*bla*Δ*mini-Tn5*	Suicide vector used for insertion mutation	Fang et al[25]

ATCC = American Type Culture Collection; NTUH = National Taiwan University Hospital.

Bioinformatics analysis

We analyzed the predicted conserved domain and compared the sequence similarity, using the National Center for Biotechnology Information protein BLAST.

Construction of *dam* mutant

Double-crossover replacement of *dam* is lethal in *K. pneumoniae*.[21] We used an established method to construct insertion mutation through single-crossover recombination.[21,26] In brief, we amplified a partial fragment (nucleotides 143—545) of *dam* from NTUH-K2044. The partial *dam* fragment was cloned to a TA cloning vector. Next, we inserted a kanamycin-resistance gene (*Km*) into the TA cloning vector, at a *Pst*I site that is distal to 3′ end of the partial *dam* fragment. Then the *Km-dam* (nucleotides 143—545) fragment was excised by *Eco*RI and inserted into the suicide vector pUTKm1Δ*tnp*Δ*bla*Δ*mini-Tn5*.[25] pUTKm1Δ*tnp*Δ*bla*Δ*mini-Tn5*::*Km-dam*(143—545) was sent into the wild type through conjugation.[24] We selected transconjugant clones on LB agar supplemented with kanamycin (50 μg/mL) and chlorhexidine (5 μg/mL), on which the chlorhexidine-sensitive donor *E. coli* S17-1/*λpir* and kanamycin-sensitive wild type *K. pneumoniae* NTUH-K2044 will not grow.

Methylation state of DNA

We determined the methylation state of DNA using restriction digestion by *Mbo*I and *Sau*3AI.[20] We used the extracted genomic DNA from wild-type *K. pneumoniae* NTUH-K2044 as negative control, and that from *E. coli* RS5033 (a *dam* mutant) as positive control.[23]

Frequency of spontaneous mutation

We determined the frequency of spontaneous mutation as previously described.[20] In brief, using a loop, we inoculated a few hundred cells from each strain into 5 mL of LB broth and then cultured the cells overnight to saturation at 37°C with shaking. We plated each culture on the LB agar, with or without rifampicin (100 μg/mL), and incubated the plates overnight at 37°C. We then scored the plates for rifampicin-resistant colonies and calculated the frequency of spontaneous mutation. The assay was repeated for a total of 12 times in each strain.

Growth curves

We diluted overnight broth culture with fresh LB broth in a 1:100 ratio. We then determined the growth curves of *dam* mutant and the wild type, using serial measurements of optical density at 600 nm at 0.5-hour intervals. The assay was repeated for a total of four times in each strain.

Colony mucoviscosity

We assayed colony mucoviscosity as previously described.[3] The mucoviscosity was measured using a loop to stretch the colony on 5% sheep blood agar plate after 37°C incubation overnight.

Serum-resistance assay

We assayed serum resistance as previously described.[3] A bacterial inoculum of 2.5×10^4 colony-forming units (CFUs), which was prepared from the mid-log phase, was mixed at a 1:3 volume/volume ratio with nonimmune human serum from healthy voluntary donors. The mixture was incubated at 37°C. Colony count was checked at baseline and then at 1 hour, 2 hours, and 3 hours after serum treatment, using the serial dilution method. *K. pneumoniae* NTUH-K5906 was used as a serum-sensitive control. We repeated the assay for a total of four times in each strain. Survival ratios (i.e., ratio between the colony count after serum treatment and that at baseline) were compared using Mann—Whitney *U* test.

Mouse inoculation

Bacterial virulence in mice was assayed as previously described.[3,7] The bacterial inocula, prepared from cultures at mid-log phase, were diluted in 100 μL of normal saline before use. For each dose of inoculum, four mice were used. Each mouse received a single inoculation via an

intraperitoneal injection or gastric ingestion (with a nasogastric tube) and was observed for up to 30 days before euthanasia by CO_2. Lethal dose, 50% (LD_{50}) was calculated using the established method by Reed and Muench.[27]

Results

Identification of the *dam* gene in *K. pneumoniae* NTUH-K2044

On the *K. pneumoniae* NTUH-K2044 genome, only one ORF designated as "KP5088" has a significant match (82% similarity) in amino acid sequence with the Dam of *E. coli* K-12. The protein encoded by ORF KP5088 is a D12 class N6 adenine-specific DNA methyltransferase, with the predicted function of Dam. The *dam* gene in *K. pneumoniae* NTUH-K2044 is 828 nt in length and encodes a protein with 275 amino acids. We deposited the sequences in GenBank under the accession number AB158489. The *dam* gene in *K. pneumoniae* MGH-78578 is nearly identical to that in *K. pneumoniae* NTUH-K2044, with only three differences in nucleotides (nucleotide 306: G to A, 360: C to T, and 744: G to T), which do not alter the predicted amino acid sequence.

dam mutant

We obtained 24 kanamycin-resistant transconjugant clones. We chose one of these potential recombinants for further characterization. Single-crossover insertion of suicide vector into the wild type will cause truncation of the *dam* gene on the mutant genome, resulting in two partial *dam* fragments with *Km*-pUTKm1Δ*tnp*Δ*bla*Δ*mini-Tn5* between them. Truncation of the *dam* gene on mutant genome was verified by the result of polymerase chain reaction (PCR), which used different combinations of primer pairs designed from nucleotide sequences of the *K. pneumoniae dam* gene, the adjacent area on the NTUH-K2044 genome, and the suicide vector. The PCR result was further confirmed by DNA sequencing of the PCR products.

Loss of Dam methylation in mutant

To confirm the loss of Dam activity in the mutant, the genomic DNA extracted from the *dam* mutant was digested using restriction enzymes *Mbo*I and *Sau*3AI. Both enzymes recognize the 4-nt restriction site GATC; however, Dam methylation blocks the digestion by *Mbo*I, and not that by *Sau*3AI. Therefore, Dam-methylated DNAs will be digested only by *Sau*3AI, whereas unmethylated DNAs will be digested by both enzymes. We found that *Mbo*I failed to digest the extracted genomic DNA from wild-type NTUH-K2044, but successfully and completely digested the extracted genomic DNA from *E. coli* RS-5033 and the *K. pneumoniae dam* mutant (Figure 1). Meanwhile, *Sau*3AI successfully digested the extracted genomic DNAs from all the three strains. These results indicate that the insertion mutation of *dam* in *K. pneumoniae* NTUH-K2044 was associated with a loss of Dam methylation on the mutant genome.

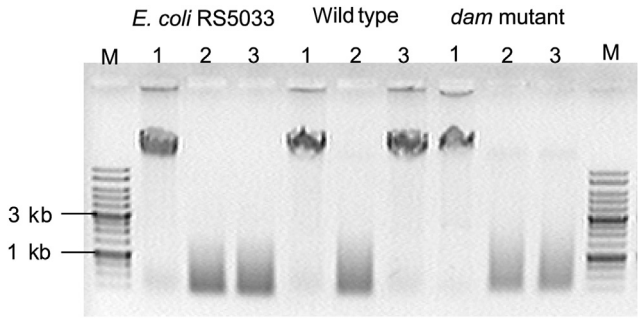

Figure 1. *Mbo*I and *Sau*3AI restriction digestion of genomic DNAs extracted from *Escherichia coli* RS5033, *Klebsiella pneumoniae* NTUH-K2044 wild type, and *dam* mutant. Lane 1 = uncut genomic DNA; Lane 2 = *Sau*3AI digestion; Lane 3 = *Mbo*I digestion; M = marker.

Because Dam is required for DNA mismatch repair, the loss of Dam activity will cause an increased frequency of spontaneous mutation. We found that the *K. pneumoniae dam* mutant had a mean spontaneous mutation frequency of $1.1 \pm 0.2 \times 10^{-5}$, whereas that of the wild type was only $1.6 \pm 0.8 \times 10^{-8}$. The 1000-fold increase in spontaneous mutation frequency further confirms the loss of Dam activity in the mutant.

Growth rate

The growth rates of *dam* mutant in the log phase was not significantly different from that of the wild type (Figure 2), although the mutant had a slightly longer lag phase before the onset of logarithmic growth.

Loss of hypermucoviscosity and serum resistance

K. pneumoniae NTUH-K2044 is characterized by hypermucoviscous colony and extremely high serum resistance. Both phenotypes are markers for clinical invasiveness.[2,28] The *dam* mutant exhibited a significant decrease in colony hypermucoviscosity compared with the wild type (length of string: 10 mm vs. 50 mm). The mutant lost at least 68% of serum resistance when compared with the wild

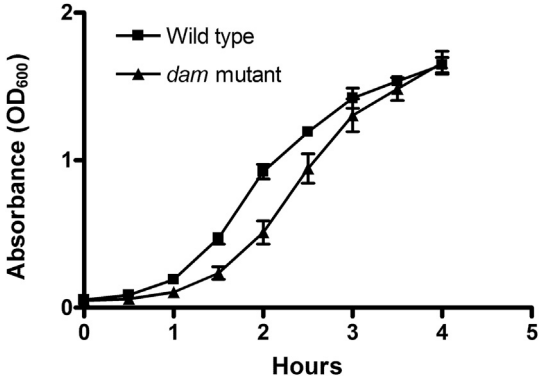

Figure 2. Growth curves of *Klebsiella pneumoniae* NTUH-K2044 wild type and the *dam* mutant. The error bars represent standard errors.

type (survival ratio at 1 hour: 2.6 ± 0.4 vs. 8.2 ± 1.9; at 2 hours: 3.9 ± 1.6 vs. 17.4 ± 3.6; p values < 0.05, Mann–Whitney U test; Figure 3).

Attenuation in mice models

Table 2 shows the result of mice inoculation tests. Compared with the wild type, virulence to mice decreased in the *dam* mutant by 40-fold in the intraperitoneal injection model (LD_{50}: 2×10^3 CFUs vs. 5×10^1 CFUs) and by sixfold in the gastric ingestion model (LD_{50}: 3×10^4 CFUs vs. 5×10^3 CFUs).

Downstream genes

Complementation with overproduction of Dam leads to attenuation,[11,16,17] because precise amounts of Dam are required for full virulence.[11] To exclude the possibility that the attenuation in the *dam* mutant might be caused by loss of downstream gene expression, we identified 10 ORFs distal to *dam* on the genome of NTUH-K2044. The three genes (*rpe*, *gph*, and *trpS*) immediately distal to *dam* are housekeeping genes that are not related to bacterial virulence (Table 3). ORFs distal to *trpS* are transcribed in the opposite direction, and therefore, are not in the same transcription unit.

Discussion

Our study shows that Dam deficiency in *K. pneumoniae* genotype K1 causes a significant reduction (i.e., ≥68% loss) in serum resistance. This finding is highly clinically relevant. The resistance to human serum is a prerequisite for bacterial hematogenous invasion of vital organ systems. Hence, a significant decrease in serum resistance has a direct impact on the capability of *K. pneumoniae* genotype K1 to cause metastatic septic ocular/CNS complications.[2] In addition, the *in vivo* virulence substantially decreased by 83–98% (with the LD_{50} in mice increasing by 6–40-fold) in the mutant. The attenuation of *dam* mutant was not explained by other factors. It was unrelated to growth rate, which was similar between the mutant and wild type. It was not caused by loss of expression of the downstream genes, which are unrelated to bacterial virulence. Our findings support the view that DNA adenine methylation plays an important role in modulating the pathogenicity of *K. pneumoniae* genotype K1.

Dam is a global regulator of virulence genes in *Salmonella*, in which the *dam* mutants are avirulent.[11] By contrast, in *K. pneumoniae* genotype K1, the *dam* mutant is incompletely attenuated, as shown in the *in vitro* serum resistance assay and *in vivo* virulence tests. Our finding suggests the existence of other regulatory factors for virulence gene expression in this hypervirulent pathogen. When there are two or more genes with similar biological functions, inactivation of only one gene may not be able to distinctly change the phenotype. For example, iron acquisition is essential for bacterial virulence.[6] In *K. pneumoniae* NTUH-K2044, there are at least four different iron-uptake systems.[7–9] Deletion of three of the four systems leads to incomplete attenuation.[9] Hence, our finding of an incomplete virulence attenuation in the *dam* mutant does not negate the importance of Dam in *K. pneumoniae* genotype K1. Instead, our result implies that multiple regulatory mechanisms—including Dam—are involved in the enhanced pathogenicity of this hypervirulent pathogen.

In addition to the biological implication, incomplete attenuation of the *dam* mutant has important implications

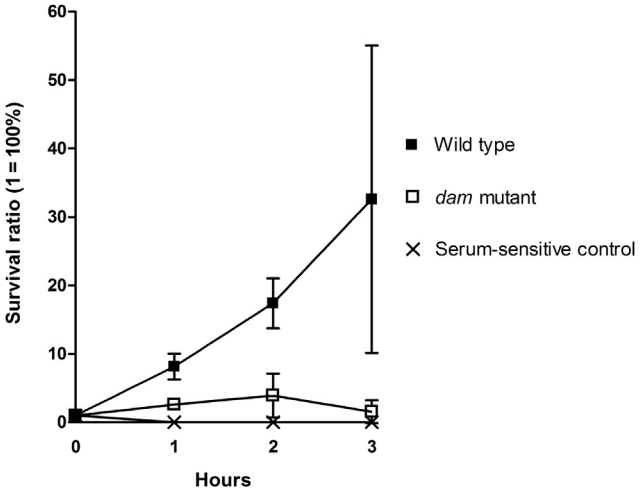

Figure 3. Serum-resistance assay of *Klebsiella pneumoniae* NTUH-K2044 wild type, the *dam* mutant, and serum-sensitive control *K. pneumoniae* NTUH-K5906. Survival ratio is the ratio between the colony count after 1-, 2-, and 3-hour serum treatment and the colony count at baseline. The error bars represent standard errors.

Table 2 Results of mice inoculation.

Inoculum (CFUs)	Survivors/inoculated no. (%)	LD_{50} (CFUs)[a]
Intraperitoneal injection		
dam mutant		2×10^3
10^2	4/4 (100)	
10^3	3/4 (75)	
10^4	0/4 (0)	
10^5	0/4 (0)	
10^6	0/4 (0)	
Wild type		5×10^1
10^1	4/4 (100)	
10^2	1/4 (25)	
Gastric ingestion		
dam mutant		3×10^4
10^4	2/4 (50)	
10^5	2/4 (50)	
Wild type		5×10^3
10^3	4/4 (100)	
10^4	1/4 (25)	

CFU = colony-forming unit; LD_{50} = lethal dose, 50%.
[a] Estimated by Reed and Muench's method,[27] which assumes that a mouse that died at a given infectious dose would also die at higher infectious dosages; likewise, a mouse that survived at a given infectious dosage would also survive at lower infectious dosages. This method allows for a more precise determination of LD_{50} from a smaller number of mice.

Table 3 Downstream genes on *Klebsiella pneumoniae* NTUH-K2044 genome, from 5′ to 3′.

Open reading frame no.[a]	GenBank accession no.	Gene (functions)
KP5087	BAH65532	*rpe* (encodes ribulose-phosphate 3-epimerase, an enzyme in Calvin cycle for carbon fixation)
KP5086	BAH65531	*gph* (encodes phosphoglycolate phosphatase, an enzyme involved in cellular DNA repair)
KP5085	BAH65530	*trpS* (encodes tryptophanyl-transfer RNA synthetase)
KP5084	BAH65529	*cysG* (encodes siroheme synthase, an enzyme in bacterial sulfur metabolism)
KP5083	BAH65528	*nirD* (encodes nitrite reductase, a small subunit)
KP5082	BAH65527	*nirB* (encodes nitrite reductase, a subunit)
KP5080	BAH65526	*codA* (encodes cytosine deaminase, an enzyme that converts cytosine to uracil)
KP5079	BAH65525	*yhfC* (a putative major facilitator superfamily transporter system involved in carbohydrate transport/metabolism)

[a] *K. pneumoniae* NTUH-K2044 genome website: http://genome.nhri.org.tw/KP/.

for the prevention and treatment of *K. pneumoniae* genotype K1 infections. First, the *dam* mutant could be unsafe for vaccine use. Second, chemical compounds inhibiting Dam activity would not be able to replace the current standards of treatment, which include drainage of abscesses and potent antibiotics such as third-generation cephalosporins.[2,29] Nevertheless, Dam-targeted novel compounds may still be considered as candidates for adjuvant therapy,[30] which could be useful particularly in the management of life-threatening or complicated infections of this highly virulent pathogen.[31]

Our finding of a positive role for Dam in *K. pneumoniae* genotype K1 is similar to that observed in non-K1 strains. In a previous study of *K. pneumoniae* K2 strain, the *dam* mutant was partially attenuated with its virulence lost at a low infectious dose (10^3 bacteria) but not at a larger infectious dose (10^8 bacteria),[21] although the level of virulence reduction could not be quantified due to a lack of data on LD_{50}. Moreover, we found that the predicted amino acid sequence of the *dam* gene from *K. pneumoniae* genotype K1 (NTUH-K2044) is identical to that of classical *K. pneumoniae* (MGH-78578, a K52 strain), which indicates a high resemblance between genotype K1 and classical *K. pneumoniae* in the structure (and possibly also function) of their Dam proteins.

Besides the important role in bacterial pathogenicity, Dam is also essential for bacterial DNA mismatch repair.[10] In Dam-deficient and Dam-overproducing mutants, the spontaneous mutation rate significantly increased: for example, by eightfold in *P. multocida*[18]; by 20—40-fold in *E. coli* and *Yersinia enterocolitica*,[32,33] and by up to 1000-fold in *S. flexneri*.[20] In our study, the *dam* mutant of *K. pneumoniae* NTUH-K2044 also had a 1000-fold increase in spontaneous mutation rate. Previous studies have demonstrated that Dam does not affect bacterial pathogenicity through the increase in mutation rate.[11,20] In *Salmonella*, the *mutS* mutant is not attenuated in virulence assays despite a 40—500-fold increase in spontaneous mutation.[11] Likewise, in *S. flexneri*, the *dam* mutant shows no significant attenuation even with a 1000-fold increase in spontaneous mutation.[20] However, an increased mutation rate does facilitate the development of bacterial resistance to antimicrobial agents.[34] This issue is particularly important for *K. pneumoniae*, which has a high colonization rate in hospitalized patients and continues to be a common pathogen for a variety of nosocomial infections.[1] The markedly increased mutation rate in *K. pneumoniae dam* mutant raises serious concerns for accelerated emergence of antibiotic resistance, which needs to be addressed in the development of novel chemical compounds targeted at inhibiting bacterial Dam.[30]

The attempt to construct a double-crossover *dam* mutant with allelic replacement was unsuccessful for *K. pneumoniae* NTUH-K2044 in our study. Failures to construct this type of mutants have also been reported for non-K1 *K. pneumoniae*, which were attributed to lethal effects of such a mutation.[21] On the other hand, the single-crossover mutant that we obtained did exhibit characteristics consistent with the loss of *dam* function, including a loss of DNA adenine methylation in genomic DNA and a 1000-fold increase in spontaneous mutation rate. However, the single-crossover mutants may revert to wild type in settings devoid of kanamycin, such as the *in vivo* mice inoculation tests. As the wild type is highly virulent to mice (with an LD_{50} of 50 CFUs), even a few CFUs of revertants in mice can lead to an underestimation of LD_{50} for the mutant. Hence, the actual difference between *dam* mutant and the wild type in LD_{50} might have been larger (and thus the impact of Dam might have been greater) than that observed in our study.

In conclusion, our results support that DNA adenine methylation plays an important role in modulating the pathogenicity of *K. pneumoniae* genotype K1. Attenuation of the *dam* mutant is not complete, which suggests the existence of other regulatory factors for virulence gene expression in this hypervirulent pathogen.

Conflicts of interest

All authors reported no conflict of interest.

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