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## DNA adenine methylase is involved in the pathogenesis of *Edwardsiella tarda*

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### ARTICLE INFO

#### Article history:

Received 23 March 2009

Received in revised form 23 August 2009

Accepted 4 September 2009

#### Keywords:

Antisense RNA

DNA adenine methylase

*Edwardsiella tarda*

RNA interference

Virulence

### ABSTRACT

*Edwardsiella tarda* is a serious aquaculture pathogen that can infect many cultured fish species. The aim of this study was to investigate the potential importance of DNA adenine methylase (Dam) in *E. tarda* pathogenesis. The *E. tarda* *dam* gene (*dam<sub>Et</sub>*) was cloned from a pathogenic strain, TXD1, isolated from diseased fish. *Dam<sub>Et</sub>* shares high (70.2%) sequence identity with the Dam proteins of *Yersinia enterocolitica* and several other bacterial species. Recombinant *Dam<sub>Et</sub>* is able to complement a *dam*-deficient *Escherichia coli* strain and methylate the genomic DNA. Attenuation of *dam<sub>Et</sub>* expression by antisense RNA interference had no apparent effect on the growth of TXD1, but caused significant attenuation of overall bacterial virulence and altered several stress responses including spontaneous mutation, recovering from UV radiation and H<sub>2</sub>O<sub>2</sub> exposure, binding to host mucus, and dissemination in host blood and liver. In addition, attenuation of *dam<sub>Et</sub>* expression increased *luxS* expression and AI-2 activities in *E. tarda*. These results indicate that *Dam<sub>Et</sub>* is a virulence determinant and plays a role in the pathogenesis of TXD1, and that temporal expression of *dam<sub>Et</sub>* is essential for optimal bacterial infection.

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### 1. Introduction

*Edwardsiella tarda* is a Gram-negative bacterium of the family *Enterobacteriaceae*. It is an opportunistic pathogen of humans, animal, and fish. *E. tarda* can infect a wide range of fish species and cause edwardsiellosis, a systematic disease that often leads to mortality. A number of virulence systems have been identified in *E. tarda*, notably the type III secretion system (TTSS) (Tan et al., 2005), the type VI secretion system (T6SS) (Zheng and Leung, 2007), and the EthA/EthB haemolysin system (Hirono et al., 1997). In addition, recent studies have found that the LuxS/AI-2 quorum sensing system is also involved in the pathogen-

esis of *E. tarda* and that a virulence-associated protein, EthR, regulates the expression of both the LuxS/AI-2 quorum sensing system and the EthA/EthB haemolysin system (Zhang et al., 2008a; Wang et al., 2009a).

DNA adenine methylase (Dam) is an enzyme that catalyzes the methylation of N-6 of the adenine residue in GATC sequences. Dam is known to function in multiple cellular processes, including DNA replication, gene expression, and mismatch repair (Casades and Low, 2006; Lobner-Olesen et al., 2005). Methyl-directed DNA mismatch repair system corrects base mismatches by cleaving, via the endonuclease MutH, the unmethylated daughter strand at a nearby GATC site. Overproduction of Dam is known to cause untimely methylation of the daughter strand and prevent the cleavage of MutH. As a result, strains overexpressing *dam* exhibit enhanced mutation frequency. On the other hand, *dam* mutation can also hamper the action of the mismatch repair system (Marinus, 1996). Hence, in the

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**Table 1**  
Bacterial strains, plasmids, and primers used in this study.

Strain or plasmid or primer	Relevant characteristics <sup>a</sup>	Source or reference
<b>Bacterial strains</b>		
<i>Edwardsiella tarda</i> TXD1	Rif <sup>R</sup> ; fish pathogen	Sun et al. (2009)
<i>Escherichia coli</i> DH5 $\alpha$ ER2925	F <sup>-</sup> ( $\Phi$ 80d <i>lacZ</i> $\Delta$ M15) $\Delta$ ( <i>lacZYA-argF</i> ) U169 <i>recA1 endA hsdR17 (rk<sup>-</sup>, mk<sup>-</sup>) supE44 thl dam13::Tn9 xylA-5 mtl-1 thi-1 mcrB1</i>	Takara, Dalian, China New England Biolabs
<b>Plasmids</b>		
pBS-T	Ap <sup>R</sup> ; cloning vector	Tiagen, Beijing, China
pBSDP	Ap <sup>R</sup> ; carrying partial <i>dam<sub>Et</sub></i>	This study
pBT	Ap <sup>R</sup> ; cloning vector	Zhang et al. (2008a)
pBTDAM	Ap <sup>R</sup> ; carrying <i>dam<sub>Et</sub></i>	This study
pBTDI	Ap <sup>R</sup> ; carrying antisense <i>dam<sub>Et</sub></i>	This study
pJDAM	Ap <sup>R</sup> ; carrying <i>dam<sub>Et</sub></i>	This study
pJDRI	Ap <sup>R</sup> ; carrying antisense <i>dam<sub>Et</sub></i>	This study
pJRA	Ap <sup>R</sup> ; broad host range plasmid	Zhang et al. (2008a)
<b>Primers</b>		
	Sequences (5' $\rightarrow$ 3') <sup>b</sup>	
DAMF2	CGCGCTTTTGGAAATGGGC	
DAMR1	GTGCATAAGGCGGATCGCA	
DAMF10	<u>GATATCGCCCGTTGAGCCAG</u> (EcoRV)	
DAMR9	<u>GATATCTTAACGCTTGCCGTACA</u> (EcoRV)	
ECF3	CTGGATAAGGTTGATGGGAATC	
ECR3	TGCCGCTCCATCGTGCA	
EGF1	GACGCCCTGAGCCAAAG	
EGR2	CCTGGCAGCCACACCGTA	

<sup>a</sup> Ap<sup>R</sup>: ampicillin resistant; Rif<sup>R</sup>: rifampicin resistant.

<sup>b</sup> Underlined nucleotides are restriction sites of the enzymes indicated in the brackets at the ends.

natural state of the cell, the number of Dam molecules is maintained at a controlled level (Boye et al., 1992).

In addition to participate in basic biological processes, Dam is also known to be a virulence factor in many pathogenic prokaryotes, and either its overexpression or mutation reduces the infectivity of the bacteria (Erova et al., 2006; Falker et al., 2007; Mehling et al., 2007; Oza et al., 2005; Pouillot et al., 2007).

In this study, we describe the cloning and analysis of the *dam* gene from a pathogenic *E. tarda* strain isolated from diseased fish. We found that interference with *dam* expression had drastic effects on bacterial capacities that are associated with infection.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. All strains were grown in Luria-Bertani broth (LB) medium (Sambrook et al., 1989) at 37 °C (for *Escherichia coli*) or 28 °C (for *E. tarda*). When appropriate, ampicillin and rifampicin were added at 50 and 30  $\mu$ g/ml, respectively.

### 2.2. Examination of the methylation status of TXD1

The genomic DNA of TXD1 was prepared as described previously (Zhang and Sun, 2007) and digested with the restriction endonucleases DpnI, DpnII, and Sau3A1, respectively. All these enzymes have the sequence GATC as target site, but DpnI recognizes and cuts the target only when it is methylated, whereas DpnII cuts only when the target is unmethylated and Sau3A1 cuts both methylated and unmethylated targets.

### 2.3. Cloning of *dam<sub>Et</sub>*

An internal segment of *dam<sub>Et</sub>* was cloned from TXD1 by degenerate PCR using primers DAMF2/DAMR1 (Table 1), which were designed based on the conserved sequences of known *dam* genes. The PCR products were ligated into pBS-T, resulting in pBSDP, which was subjected to DNA sequencing. Primers specific to *dam<sub>Et</sub>* were designed based on the sequencing result of pBSDP and used to obtain the complete sequence of *dam<sub>Et</sub>* by genome walking as described previously (Zhang and Sun, 2007).

### 2.4. Plasmid construction

The plasmids and primers used in this study are listed in Table 1. All PCR amplifications were performed with Pfu DNA polymerase (Tiagen, Beijing, PR China) to generate PCR products with blunt ends. To construct pJDAM, *dam<sub>Et</sub>* was amplified by PCR with primers DAMF10/DAMR9, and the PCR products were inserted into pBT at the SmaI site; the resulting plasmid pBTDAM was digested with ScaI, and the fragment carrying *dam<sub>Et</sub>* was inserted into pJRA at the EcoRV site, resulting in pJDAM. To construct pJDRI, the DNA corresponding to the region between positions 820 and –126 relative to the translational start of *dam<sub>Et</sub>* was amplified by PCR with primers DAMR9/DAMF10; the PCR products were inserted into pBT at the SmaI site so that antisense *dam<sub>Et</sub>* is constitutively expressed under the *trc* promoter (Zhang et al., 2008a); the resulting plasmid pBTDI was digested with ScaI, and the fragment carrying antisense *dam<sub>Et</sub>* was inserted into pJRA at the EcoRV site, yielding pJDRI. pJDRI and pJRA were conjugated into TXD1 as described previously (Zhang et al., 2008a).

## 2.5. Examination of complementation between *dam*<sub>Et</sub> and the *E. coli dam*

pJDAM and the control plasmid pJRA were introduced separately into ER2925 by transformation, resulting in transformants ER2925/pJDAM and ER2925/pJRA, respectively. The genomic DNA of ER2925/pJDAM and ER2925/pJRA were extracted and digested with DpnI, DpnII, and Sau3A1, respectively.

## 2.6. Quantitative real time reverse transcriptase PCR (qRT-PCR)

For the analysis of antisense *dam*<sub>Et</sub> expression, TXD1/pJDRI and TXD1/pJRA were cultured in LB medium to OD<sub>600</sub> of 0.3, 0.6, 1, and 1.3. For the analysis of the expression of virulence-associated genes, TXD1/pJDRI and TXD1/pJRA were cultured in LB medium to OD<sub>600</sub> of ~0.9. Total RNA was extracted from cells by using the RNeasy Tissue/Bacteria Kit (Qiagen, Beijing, PR China). qRT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems, USA) by using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, PR China) as described previously (Zhang et al., 2008b). Each assay was performed in triplicate with 16S rRNA as controls. The primers used for qRT-PCR of 16S rRNA, *eseB*, *eseD*, *esrA*, *orf26*, *ethA*, *ethB*, and *luxS* were described previously (Zhang et al., 2008a); the primers used for qRT-PCR of *evpC* and *evpG* are ECF3/ECR3 and EGF1/EGR2 respectively. All data are given in terms of relative mRNA, expressed as means plus or minus standard errors of the means (SE). Statistical analyses were performed using Student's *t* test.

## 2.7. Antisense RNA interference

Antisense RNA interference was performed by introducing the plasmid pJDRI, which constitutively expresses antisense *dam*<sub>Et</sub> under the *trc* promoter, into TXD1 via conjugation as described previously (Sun et al., 2009), resulting in transconjugant TXD1/pJDRI. As a control, the plasmid pJRA was also introduced into TXD1 via conjugation, resulting in transconjugant TXD1/pJRA. *dam*<sub>Et</sub> expression in TXD1/pJDRI and TXD1/pJRA was subsequently examined by qRT-PCR.

## 2.8. Biofilm assay

Cells were cultured in LB medium to exponential phase and diluted to 10<sup>5</sup> CFU/ml. The diluted cells were transferred into a 96-well polystyrene plate (Nunc, Denmark) and incubated at 28 °C for 24 h without agitation. After the incubation, the unattached cells were removed from the wells, and the wells were washed rigorously with PBS. The attached cells were treated with Bouin fixative for 1 h and stained with 1% crystal violet solution for 20 min. The samples were then measured for absorbance at A<sub>570</sub>.

## 2.9. UV and H<sub>2</sub>O<sub>2</sub> sensitivity analysis

Cells were cultured in LB medium at 28 °C to an OD<sub>600</sub> of 0.4 and diluted 2× in fresh LB medium. Two milliliters of

the cells were transferred to a Petri dish and exposed to UV radiation. Viable cells were determined by plating on LB agar plates, and colonies were counted after incubation at 28 °C for 48 h. Statistical analysis was performed using one-way analysis of variance (ANOVA).

H<sub>2</sub>O<sub>2</sub> treatment and survival analysis were performed exactly as described previously (Sun et al., 2008). Statistical analysis was performed by using ANOVA.

## 2.10. Determination of MIC and spontaneous mutation frequency

The minimum inhibitory concentrations (MIC) of rifampicin, chloramphenicol, kanamycin, and tetracycline were determined as described previously (Sun et al., 2009). To determine spontaneous mutation frequencies, cells were cultured in LB medium at 28 °C to an OD<sub>600</sub> of 1 and harvested by centrifugation. 10<sup>10</sup> cells were plated on LB agar plates containing chloramphenicol (30 µg/ml), kanamycin (30 µg/ml), and tetracycline (20 µg/ml) respectively. The plates were incubated at 28 °C for 48 h, and the colonies that emerged were counted. Mutation frequencies were calculated by dividing the numbers of antibiotic-resistant colonies by the numbers of total viable cells.

## 2.11. Fish

Healthy Japanese flounder (*Paralichthys olivaceus*) were purchased from a commercial fish farm (Rizhao, Shandong, PR China) and maintained at 20–22 °C in aerated seawater that was changed twice daily. Fish were fed daily with commercial dry pellets and anaesthetized with tricaine methanesulfonate (MS-222, Sigma) prior to experiments involving injection, blood collection, or sacrifice. Sacrifice was performed by euthanizing the fish with an overdose of MS-222 as described previously (Wang et al., 2009b). All experiments involving animals were carried out in accordance with relevant government regulations.

## 2.12. Mucus binding assay

Skin mucus was prepared by rubbing the mucus from the body surface of healthy Japanese flounder (average 620 g). To prepare intestinal mucus, Japanese flounder were starved for one week to allow the food in the gut to be processed. The intestines of the fish were removed under aseptic conditions and split open with a scalpel. Mucus in the intestinal lumen was collected with a plastic spatula and suspended in PBS. The intestinal and skin mucus were centrifuged twice at 12,000 × g for 20 min, and the supernatant was filtered through 0.45-µm pore-size filter membranes. The concentration of the mucus protein was determined by the Bradford method and adjusted to 1 mg/ml. Binding of bacterial cells to fish mucus was determined using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Beijing, PR China) with bovine serum albumin (BSA) as non-specific binding control and PBS as negative control. Relative binding rate was calculated according to the following formula: {(absorbance of mucus-bound cells – absorbance of BSA-bound cells)/absorbance of negative control} × 100%.

### 2.13. Bacterial dissemination in fish blood and liver

TXD1/pJDRI and TXD1/pJRA were cultured to mid-logarithmic phase and resuspended in PBS to  $5 \times 10^7$  CFU/ml. Japanese flounder (~11 g) were divided randomly into two groups (five fish/group) and injected intraperitoneally (i.p.) with 100  $\mu$ l of TXD1/pJDRI or TXD1/pJRA. Blood and liver were taken aseptically from the fish at 18 h post-infection. The livers were homogenized in PBS. After serial dilutions, the homogenates and blood were plated in triplicate on LB agar plates supplemented with ampicillin. After incubation at 28 °C for 48 h, the colonies that appeared on the plates were enumerated. The genetic nature of these colonies was verified by PCR analysis using primers specific to TXD1/pJDRI and TXD1/pJRA. The PCR products were randomly selected for DNA sequencing. Statistical analyses were performed using Student's *t* test.

### 2.14. Plasmid stability analysis

The stability of TXD1/pJDRI and TXD1/pJRA under in vivo conditions during the infection process was determined as described previously (Zhang et al., 2008a).

### 2.15. Virulence determination

TXD1/pJDRI and TXD1/pJRA were cultured to mid-logarithmic phase and resuspended in PBS to  $5 \times 10^7$  CFU/ml. Japanese flounder (~11 g) were divided randomly into three groups (30 fish/group) and injected i.p. with 100  $\mu$ l of TXD1/pJDRI, TXD1/pJRA, and PBS respectively. The fish were monitored for mortality over 20 days post-infection. Statistical analyses were performed using Chi-square test.

### 2.16. AI-2 assay

AI-2 assay was performed as described previously (Zhang et al., 2008a).

### 2.17. Nucleotide sequence accession number

The nucleotide sequence of *dam*<sub>Et</sub> has been deposited in the GenBank database under the accession number FJ810878.

## 3. Results

### 3.1. Identification of the *dam* gene from TXD1

The results of DpnI, DpnII, and Sau3AI digest showed that the genomic DNA of *E. tarda* TXD1 was resistant to cleavage by DpnII but not by DpnI or Sau3AI, suggesting that TXD1 possesses a functional *dam* gene. The *dam* gene of TXD1 (named *dam*<sub>Et</sub>) was subsequently cloned by PCR amplification with degenerate primers. *dam*<sub>Et</sub> encodes a putative protein of 272 amino acids that shares the highest (70.2%) overall sequence identity with the Dam proteins of *Yersinia enterocolitica* subsp. *enterocolitica*, *Erwinia carotovora* subsp. *atroseptica*, *Y. pseudotuberculosis*, *Y. pestis*, and *Serratia proteamaculans*.

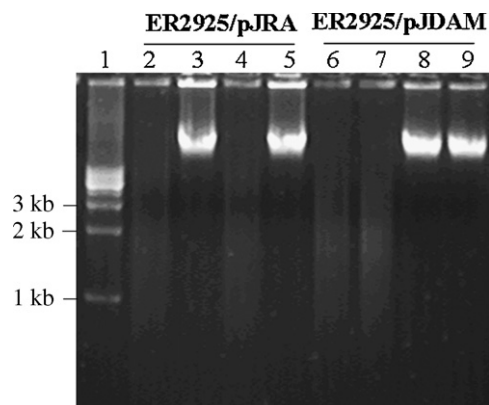


Fig. 1. Restriction endonuclease digestion of ER2925/pJRA and ER2925/pJDAM. Genomic DNA of ER2925/pJRA and ER2925/pJDAM were digested with Sau3A1 (lanes 2 and 6), DpnI (lanes 3 and 7), and DpnII (lanes 4 and 8) respectively. After digestion, the samples were subjected to electrophoresis in a 0.8% agarose gel. Lanes 5 and 9, uncut DNA of ER2925/pJRA and ER2925/pJDAM respectively; lane 1, DNA markers.

### 3.2. Complementation between *dam*<sub>Et</sub> and the *E. coli* *dam*

The results of DpnI, DpnII, and Sau3AI digest showed that the genomic DNA of ER2925/pJDAM was resistant to DpnII but sensitive to DpnI and Sau3AI; in contrast, the genomic DNA of ER2925/pJRA was resistant to DpnI but sensitive to DpnII and Sau3AI (Fig. 1). These results demonstrate that *dam*<sub>Et</sub> encodes a functional adenine methylase that can methylate the chromosomal DNA of *E. coli*.

### 3.3. Interfering with *dam*<sub>Et</sub> expression by antisense RNA

A TXD1 variant, TXD1/pJDRI, was constructed, in which *dam*<sub>Et</sub> expression was attenuated by antisense RNA interference. qRT-PCR analyses indicated that the expression of antisense *dam*<sub>Et</sub> in TXD1/pJDRI was constant and not affected by growth phase, and that *dam*<sub>Et</sub> expression in TXD1/pJDRI was 4.3-fold lower than that in TXD1/pJRA. Consistently, the genomic DNA of TXD1/pJDRI exhibited partial resistance against DpnI digest (Supplementary Figure S1), suggesting the existence in TXD1/pJDRI of DNA that was unmethylated or incompletely methylated.

### 3.4. Analysis of the effects interfering with *dam*<sub>Et</sub> expression

#### 3.4.1. Effect on growth

Growth studies showed that *E. tarda* strains TXD1/pJDRI and TXD1/pJRA exhibited similar growth profiles when cultured in LB medium and produced similar amounts of biofilm on polystyrene surface. Hence, interference with *dam*<sub>Et</sub> expression has no apparent effect on planktonic or biofilm growth of TXD1.

#### 3.4.2. Effect on survival after UV radiation and H<sub>2</sub>O<sub>2</sub> treatment

The results of UV and H<sub>2</sub>O<sub>2</sub> survival analysis showed that after UV radiation, the survival rate (18%) of TXD1/pJDRI was significantly (*P* < 0.01) lower than that (40%) of

TXD1/pJRA. Similarly, the number (4.3%) of TXD1/pJDRI that survived H<sub>2</sub>O<sub>2</sub> treatment was significantly ( $P < 0.01$ ) lower than that (50.6%) of TXD1/pJRA.

#### 3.4.3. Effect on spontaneous mutation frequency

*E. tarda* TXD1 is naturally resistant against rifampicin (MIC > 20 µg/ml) and sensitive to chloramphenicol, kanamycin, and tetracycline (MIC values less than 10 µg/ml). Spontaneous mutation analyses showed that the numbers of spontaneous chloramphenicol-, kanamycin-, and tetracycline-resisting mutants that emerged from 10<sup>10</sup> CFU of TXD1/pJDRI were 162, 130, and 221 respectively, which yield spontaneous mutation rates of 1.6, 1.3, and  $2.2 \times 10^{-8}$  respectively. In contrast, no spontaneous chloramphenicol-, kanamycin-, or tetracycline-resistant mutants emerged from 10<sup>10</sup> CFU of TXD1/pJRA. Hence, the spontaneous mutation frequency of TXD1/pJDRI is more than 100-fold higher than that of TXD1/pJRA.

#### 3.4.4. Effect on binding to host mucus and dissemination in host blood and liver

Since *E. tarda* TXD1 is a fish pathogen, we examined the effect of reduced *dam<sub>Et</sub>* expression on the capacities of TXD1 that are known to be associated with infectivity, which include binding to fish mucus and dissemination in fish blood and tissues. Mucus-binding analyses showed that the numbers of TXD1/pJDRI bound to the skin and intestinal mucus of Japanese flounder were, respectively, 52- and 47-fold lower than those of TXD1/pJRA. Dissemination analyses showed that the numbers of TXD1/pJDRI recovered from the blood and liver of TXD1/pJDRI-infected were, respectively, 70- and 90-fold lower than those of TXD1/pJRA recovered from the blood and liver of TXD1/pJRA-infected fish. Plasmid stability analyses indicated that 89–91% of the colonies recovered from the blood and liver of TXD1/pJDRI-infected fish harbored pJDRI, whereas 90–91% of the colonies recovered from the blood and liver of TXD1/pJRA-infected fish harbored pJRA. Hence, during the infection process, the plasmid loss rate of TXD1/pJDRI was comparable to that of TXD1/pJRA. These results suggest that the difference between TXD1/pJDRI and TXD1/pJRA in blood/tissue dissemination is not due to difference in plasmid stability.

#### 3.4.5. Effect on overall bacterial virulence

Bacterial virulence analyses indicated that the accumulated mortality (20%) of the fish infected with TXD1/pJDRI was significantly ( $P < 0.01$ ) lower than that (86.7%) of the fish infected with TX1/pJRA, suggesting that interference with *dam<sub>Et</sub>* expression significantly attenuated overall bacterial virulence.

#### 3.4.6. Effect on the expression of virulence-associated genes and the LuxS/AI-2 quorum sensing system

qRT-PCR was carried out to determine the expression of the TTSS genes *eseB*, *eseD*, *esrA*, and *orf26*, the T6SS genes *evpC* and *evpG*, the haemolysin genes *ethA* and *ethB*, and the LuxS gene. The results showed that the expression levels of all the examined genes except *luxS* were comparable between TXD1/pJDRI and TXD1/pJRA. However, *luxS* expression was 9-fold higher in TXD1/pJDRI than

in TXD1/pJRA. Since our previous study has indicated that LuxS regulates AI-2 activity (Zhang et al., 2008a), we determined the AI-2 levels of TXD1/pJDRI and TXD1/pJRA, and the results showed that the AI-2 activity of TXD1/pJDRI was 5-fold higher than that of TXD1/pJRA.

## 4. Discussion

In *Neisseria meningitidis* and *Salmonella typhimurium*, lack of Dam-mediated methylation results in a mutator phenotype that is characterized by a high rate of spontaneous mutation (Bucci et al., 1999; Mohler et al., 2008; Torreblanca and Casadesus, 1996). In our study, we found that interference with *dam<sub>Et</sub>* expression drastically increased the spontaneous mutation frequency in the TXD1 strain of *E. tarda*. It is likely that interference with *dam<sub>Et</sub>* expression reduces the amount of Dam<sub>Et</sub> molecules in TXD1/pJDRI to a level that is lower than that required for timely methylation of the hemimethylated GATC sites occurring behind the replication fork, which is consistent with the observation that TXD1/pJDRI was partially resistance against DpnI. The unmethylated double strand DNA of TXD1/pJDRI, which, owing to the lack of methylated GATC markers that serve to discriminate the template strand from the newly synthesized strand, will impede the correction of mismatched bases.

It is known that for many bacteria, Dam mutants are sensitive to exposure to UV and other mutagenic agents that cause lesions in DNA (O'Reilly and Kreuzer, 2004; Ostendorf et al., 1999; Suter and Lovett, 2006). Similarly, we found that TXD1/pJDRI, in which *dam<sub>Et</sub>* expression was reduced by RNA interference, was impaired in the ability to recover from UV radiation and H<sub>2</sub>O<sub>2</sub> treatment, both of which induce DNA damage. These results, together with the observation that TXD1/pJDRI exhibited enhanced spontaneous mutation rate, suggest that in TXD1, repairing of DNA lesions caused by UV radiation and H<sub>2</sub>O<sub>2</sub> exposure relies on methyl-directed mismatch repair system, which in turn relies on Dam<sub>Et</sub> for proper function.

Numerous studies have demonstrated a close link between Dam and bacterial virulence (Balbontin et al., 2006; Heusipp et al., 2007; Marinus and Casadesus, 2009). Dam can affect bacterial infectivity directly by modulating the expression of virulence-associated genes (Low et al., 2001). One of the best known examples of Dam-regulated gene expression is the *E. coli pap* operon, which possesses GATC sites at the promoter region that overlap the binding sites of the regulatory protein Lrp. As such, Dam methylation affects the binding of Lrp and consequently the expression of the *pap* operon (Braaten et al., 1994; Hernday et al., 2003; Peterson and Reich, 2006, 2008). In our study, we found that compared to TXD1/pJRA, TXD1/pJDRI is drastically vitiated in pathogenicity. Although the expressions of the selected virulence genes were comparable between TXD1/pJDRI and TXD1/pJRA, the expression of *luxS* was significantly higher in TXD1/pJDRI. Our previous studies have suggested that in *E. tarda*, LuxS participates in both central metabolism and AI-2-mediated signal transduction pathway; interference with the former function of LuxS causes defect in basic physiology, whereas interference with the latter function of LuxS impairs the

development/maintenance of bacterial virulence (Zhang et al., 2008a). Since interference with *dam*<sub>Et</sub> expression enhanced *luxS* expression and AI-2 production in TXD1, it is possible that the effect of Dam on virulence is at least in part mediated by the LuxS/AI-2 quorum sensing system.

In conclusion, the results of this study demonstrate that interference with *dam*<sub>Et</sub> expression affects the ability of *E. tarda* to survive under stress conditions encountered both in vitro and in vivo the animal host; it is possible that some of the effects of Dam<sub>Et</sub> on bacterial virulence is mediated through the LuxS/AI-2 quorum sensing system, which appears to be negatively modulated by Dam<sub>Et</sub>.

## Acknowledgements

We thank Professor John R. Murphy for language assistance. This work was supported by the National Natural Science Foundation of China (NSFC) grant 40676090 and the 863 High Technology Project grant 2008AA092501.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.09.004.

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