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DNA adenine methyltransferase (Dam) controls the expression of the cytotoxic enterotoxin (act) gene of *Aeromonas hydrophila* via tRNA modifying enzyme-glucose-inhibited division protein (GidA)

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Abstract

Aeromonas hydrophila is both a human and animal pathogen, and the cytotoxic enterotoxin (Act) is a crucial virulence factor of this bacterium because of its associated hemolytic, cytotoxic, and enterotoxic activities. Previously, to define the role of some regulatory genes in modulating Act production, we showed that deletion of a glucose-inhibited division gene (*gidA*) encoding tRNA methylase reduced Act levels, while overproduction of DNA adenine methyltransferase (Dam) led to a concomitant increase in Act-associated biological activities of a diarrheal isolate SSU of *A. hydrophila.* Importantly, there are multiple GATC binding sites for Dam within an upstream sequence of the *gidA* gene and one such target site in the *act* gene upstream region. We showed the *dam* gene to be essential for the viability of *A. hydrophila* SSU, and, therefore, to better understand the interaction of the encoding genes, Dam and GidA, in act gene regulation, we constructed a *gidA* in-frame deletion mutant of *Escherichia coli* GM28 (*dam*$^{+}$) and GM33 (Δ*dam*) strains. We then tested the expressional activity of the *act* and *gidA* genes by using a promoterless pGlow-TOPO vector containing a reporter green fluorescent protein (GFP). Our data indicated that in GidA^{+} strains of *E. coli*, constitutive methylation of the GATC site(s) by Dam negatively regulated *act* and *gidA* gene expression as measured by GFP production. However, in the Δ*gidA* strains, irrespective of the presence or absence of constitutively active Dam, we did not observe any alteration in the expression of the *act* gene signifying the role of GidA in positively regulating Act production. To determine the exact mechanism of how Dam and GidA influence Act, a real-time quantitative PCR (RT-qPCR) assay was performed. The analysis indicated an increase in *gidA* and *act* gene expression in the *A. hydrophila* Dam-overproducing strain, and these data matched with Act production in the *E. coli* GM28 strain. Thus, the extent of DNA methylation caused by constitutive versus overproduction of Dam, as well as possible conformation of DNA influence the expression of *act* and *gidA* genes in *A. hydrophila* SSU. Our results indicate that the *act* gene is under the control of both Dam and GidA modification methylases, and Dam regulates Act production via GidA.

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GATC Dam target sites; Promoter activity; tRNA uridine 5 carboxymethylaminomethyl; modification enzyme

1. Introduction

Among various *Aeromonas* species, *A. hydrophila* is an aquatic environmental and food-borne microorganism which poses a health risk (Edberg et al., 2007). A diarrheal isolate SSU of *A. hydrophila* studied in our laboratory is involved in human infections, and its major virulence factor, the cytotoxic enterotoxin (Act), leads to septicemia, gastroenteritis and mortality in a mouse model (Chopra and Houston, 1999; Sha et al., 2001, 2002). We reported that a glucose-inhibited division protein, GidA, modulated the expression of the *act* gene (Sha et al., 2004).

GidA protein was first described in the *Escherichia coli* K12 strain, and disruption of the *gidA* gene affected cell division when grown in a medium containing glucose by interrupting chromosomal replication, resulting in a cell elongation phenotype (von Meyenburg et al., 1982). Further studies showed that GidA is a flavin adenine dinucleotide (FAD) binding enzyme (White et al., 2001) and also a tRNA modification methylase that catalyzes the addition of the carboxymethylaminomethyl group onto the C5 carbon atom of uridine at position 34 (U_{34}) of RNAs (Urbonavicius et al., 2005; Yim et al., 2006). It was found that the post-transcriptional modification of tRNA represents a significant element controlling gene expression (Gustilo et al., 2008).

The deletion of a *gidA* gene attenuated the pathogenesis of some bacteria, such as *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium (Cho and Caparon, 2008; Gupta et al., 2009; Shippy et al., 2011). In particular, it was noted that *gidA* gene mutant had a nearly normal global transcription profile, when compared to that of parental *S. pyogenes* strain, but the mutant produced significantly reduced levels of multiple virulence factors due to impaired translational efficiencies (Cho and Caparon, 2008). The *gidA* mutation in a plant pathogen *P. syringae* led to pleiotropic effects, resulting in diverse phenotypic traits, swarming, presence of fluorescent pigment and virulence, which indicated a possible regulatory role for GidA in moderating translational fidelity (Kinscherf and Willis, 2002).

On the other hand, DNA adenine methyltransferase (Dam) methylates the adenine base of 5′-GATC-3′ specific sites and is a global gene regulator modifying DNA. Thus, Dam plays an important role in controlling various processes in prokaryotic cells, such as transcriptional regulation, mismatch repair, host–pathogen interactions, and binding of the replication initiation complex to the methylated origin of replication (*oriC*) site (Casadesus and Low, 2006; Chatti and Landoulsi, 2008; Low and Casadesus, 2008; Marinus and Casadesus, 2009). It is known that initiation of replication of the chromosome occurs at a unique site, namely the *oriC*. Among the promoters possibly involved in the transcriptional activation of replication initiation included those for the *gidAB* genes, as transcription of *gid* genes was needed for activation of *oriC* (Bates et al., 1997). Further, regulation of DNA methylation activity through promoter methylation and compatibility between methylation of a promoter with its active transcription have been reported (Barnard et al., 2004; Marinus and Casadesus, 2009). Dam methylation can control promoter transcription, and transcriptional repression by Dam appears to be more common than transcriptional activation.

Our previous results showed that overproduction of Dam in *A. hydrophila* SSU increased Act-associated biological activities; however, a decrease in such activities was noted in the *gidA* gene mutant of the corresponding parental strain which produced a constitutive level of Dam (Erova et al., 2006a, 2006b). These data indicated that the extent of DNA methylation which was governed by the amount of Dam present dictated the levels of GidA and Act in *A. hydrophila* SSU. Dam and GidA proteins are highly conserved in many prokaryotes, and because the *dam* gene is essential for the viability of the *A. hydrophila* SSU strain, but not of *E. coli*, this fact allowed us to use *E. coli* K12 as a model to address the hypothesis regarding *act* gene regulation by both enzymes. We show data which further our understanding on the cross-talk between the decisive regulators of gene expression, namely *dam* and *gidA*, on Act production in *A. hydrophila* SSU. Our previous data indicated that Act is the most potent virulence factor among the three enterotoxins of *A. hydrophila* SSU, and GidA and Dam are involved in its control (Erova et al., 2006a; Sha et al., 2004). In the present study, we explored how these regulatory proteins, Dam and GidA, influence Act production. These studies are important, as modulation of bacterial virulence genes in general could be under the control of Dam and GidA and needs further investigation.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *A. hydrophila* and *E. coli* cultures were grown at 37 °C in Luria–Bertani (LB) broth and LB agar plates (Sambrook et al., 1989). The antibiotics ampicillin (Ap), chloramphenicol (Cm), and rifampin (Rif) were used at concentrations of 100, 20 and 200 μg/ml, respectively.

2.2. DNA isolation and polymerase chain reaction (PCR)

Plasmid DNA was isolated by using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Genomic DNA (gDNA) was isolated by using a DNeasy Tissue Kit (Qiagen). The primers (Table 2) were synthesized by Integrated DNA Technologies, Inc., Coralville, IA. Amplified PCR products were purified by using a QIAquick PCR Purification Kit (Qiagen). PCR assays from single bacterial colonies were performed as described previously (Erova et al., 2006b).

2.3. Cloning of A. hydrophila SSU upstream regions of gidA and act genes into pGlow-TOPO promoterless vector

For assaying expressional activity and to study how methylation in upstream regions of *gidA* and *act* genes affects expression of these genes, we cloned the upstream sequences into a pGlow-TOPO promoterless vector (Invitrogen, Carlsbad, CA). Upstream sequences of the *gidA* and *act* genes of *A. hydrophila* SSU contained promoter regions, which we identified by using the SoftBerry program (www.softberry.com). To obtain PCR products corresponding to the upstream regions of these genes, Up*gidA*-N/Up*gidA*-C and Up*act*-N/U*pact*-C primers were employed (Table 2) along with gDNA of *A. hydrophila* as a template. The amplified products (517-bp and 295-bp, respectively) were cloned into a pGlow-TOPO plasmid, and the recombinant plasmids were transformed into TOP10-competent cells. Recombinant plasmids were sequenced from both strands with T7-F and green fluorescent protein (GFP)-R primers (Table 2) to examine the sequence of an inserted PCR product and to confirm the correct fusion with the gene encoding GFP. The correct recombinant plasmids, pGlow-Up*gidA* and pGlow-Up*act*, were transformed into *E. coli* GM28 and GM33 strains which either harbored *dam* and *gidA* genes or were deleted for the *dam* gene and harbored *gidA* gene (Table 1). We measured transcriptional activities of promoters as a measure of GFP production in relative fluorescence units (RFU) normalized to the total

protein concentration after subtraction of the background. The promoterless pGlow-TOPO vector alone in *E. coli* GM28 and GM33 strains was used as a control.

2.4. Construction of gidA deleted in-frame mutants of E. coli GM28 and GM33 strains

To obtain *gidA* in-frame knockout mutants, we used the λ Red recombinase system (Baba et al., 2006; Datsenko and Wanner, 2000) with some modifications. The competent *E. coli* GM28 (*dam*$^{+}$) and GM33 (Δ*dam*) cells were transformed with pKD46 plasmid carrying λ Red recombinase, and cultures were grown at 30 °C because this helper plasmid has a temperature-sensitive replication. In order to generate a PCR product for electroporation of the above transformants, we constructed primers (Table 2) that had homologous regions adjacent to a *gidA* gene and that of a pKD3 plasmid carrying the Cm^{r} gene cassette, which was flanked by FRT (flippase [FLP] recognition target) sites. To eliminate unamplified methylated template DNA, we used *Dpn*I restriction enzyme to digest purified PCR products and then electroporated them into GM28 and GM33 strains carrying pKD46 plasmid which were cultivated in the presence of 10 mM L-arabinose to induce the pKD46 λ Red expression system. To cure the pKD46 plasmid from *E. coli* cells, we incubated mixtures after electroporation for 1 h at 37 °C and selected for colonies that were Cm^{r} and Ap^{s}. To remove the Cm^{r} gene from mutants, *E. coli* GM28 and GM33 strains were transformed with pCP20 plasmid that shows temperature-sensitive (30 °C) replication and thermal (43 °C) induction of FLP synthesis. After screening at 43 °C, the identified Cm^{s} and Ap^{s} single mutant colonies without pCP20 were verified by PCR.

To verify insertion of the Cm^{r} gene cassette into chromosomal DNA of *E. coli*, Cm^{r} colonies were PCR confirmed by using two pairs of primers, UpF-Cm/UpR-Cm and DownF-Cm/DownR-Cm (Table 2). The oligos UpF-Cm and DownR-Cm were designed based on flanking sequences of the *E. coli gidA* gene on chromosomal DNA. The second pair of primers, UpR-Cm and DownF-Cm, was designed based on the Cm^{r} gene cassette sequence. As shown in Fig. IA (Supplementary data), PCR products of Up- and Down-regions corresponded to the calculated sizes of 931-bp and 777-bp, respectively. A mutant thus generated still contained the Cm^{r} cassette. To confirm the *gidA* knockout mutant without antibiotic resistance (Fig. IB, Supplementary data) after curing of pCP20 plasmid, single colonies were tested by PCR with three pairs of primers (UpF-Cm/UpR-Cm, DownF-Cm/DownR-Cm and UpF-Cm/DownR-Cm) (Table 2). Primers UpF-Cm/UpR-Cm and DownF-Cm/DownR-Cm did not amplify the DNA product, a finding which indicated that the Cm^{r} gene cassette was eliminated. As expected, when we used UpF-Cm and DownR-Cm primers, a PCR product (216-bp) was amplified which represented stretches of up- and down-stream sequences from the *gidA* gene.

2.5. Transformation and electroporation of E. coli strains

Chemical-competent *E. coli* GM28 and GM33 cells for transformation were prepared by using the Z-Competent *E. coli* transformation kit from Zymo Research, Orange, CA. Competent *E. coli* GM28 (pKD46) and GM33 (pKD46) cells for electroporation of a PCR product were prepared by using 10% glycerol, as we previously described (Erova et al., 2006b). Table 1 contains the final strains with different recombinant plasmids in either *dam*- and/or *gidA*-positive and negative backgrounds that were generated for this study.

2.6. Dam methylation status of DNA from A. hydrophila SSU and E. coli strains

To verify an identical Dam methylation status of DNA from *A. hydrophila* SSU and *E. coli* GM28 and GM33 strains, DNA was isolated from these strains and digested with *Dpn*I and *Dpn*II restriction endonucleases which recognize either methylated or unmethylated DNA (Roberts et al., 2003).

2.7. Preparation of E. coli cell extracts and a gene expression assay

To prepare cell extracts from *E. coli* GM28 and GM33 strains containing constructed recombinant plasmids, we grew the cells at 37 °C in LB medium with Ap until a middle log phase. We then harvested cells and washed them with 1× Dulbecco's phosphate-buffered saline (DPBS). The cells were disrupted by sonication on ice by using two 10-s pulses, cell extracts were recovered after centrifugation, and the total amount of protein in extracts was determined by using the Bradford protein assay (Bradford, 1976). Extracts (200 μl) were assayed in duplicate by using 96-well microtiter plates (Costar, black, transparent bottom) and read employing an FL_X800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, VT). GFP levels under the control of *act* and *gidA* promoters and upstream DNA sequences were measured with excitation at 400 nm and emission at 508 nm. Background fluorescence of the extract was determined by using *E. coli* cells with the pGlow-TOPO vector alone.

2.8. Real-time (RT)-qPCR assay

Total RNA from parental *A. hydrophila* SSU and Dam-overproducing strains containing pBAD and pBAD-dam_{AhSSU} plasmids, respectively (Erova et al., 2006b), was isolated by using the RiboPure-Bacteria Kit (Ambion, Inc., Austin, TX), and 20 μg of total RNA was processed for RT-qPCR assay. RT-qPCR was performed in the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, IN) using SYBR Green I dye (Qiagen), as previously described (Fadl et al., 2006). Sequences of primers used for amplification of the *gidA* gene, *act* gene, and endogenous control *gap-1* gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) are listed in Table 2. RT-qPCR assays were run in parallel and a fold-change value was determined by using the comparative threshold method (Livak and Schmittgen, 2001) after normalization of *gidA* and *act* genes to *gap-1*.

2.9. Cloning of the A. hydrophila *act* gene under the araBAD promoter in E. coli GM28 and GM33 strains

To delineate Act production of *A. hydrophila* in *E. coli*, pBAD-*act* recombinant plasmid (Table 1) was generated by using *act*N-*Nco*I and *act*C-*Pme*I primers (Table 2) and replacing the *Nco*I-*Pme*I fragment of pBAD/Thio-E vector under its arabinose-inducible P_{BAD} promoter. The plasmid was then subjected to transformation into *E. coli* GM28 and GM33 strains and their Δ*gidA* mutants. To induce expression of the *act* gene from recombinant plasmid, the medium was supplemented with L-arabinose (0.2%) overnight.

2.10. Measurement of hemolytic activity associated with Act of A. hydrophila SSU

The hemolytic activity associated with Act was measured by examining the release of hemoglobin from rabbit erythrocytes (Colorado Serum Co., Denver, CO) in *E. coli* strains, as described previously for *A. hydrophila* (Erova et al., 2006b). *E. coli* strains with different genotypes containing the pBAD-*act* recombinant plasmid were grown overnight in LB medium with Ap and arabinose, and cell extracts were prepared as above (section 2.7). For hemolytic activity assay, 100 μl of 1× DPBS was added to each of the wells of a 96-well microtiter plate. The cell extracts were added to the first well in each row of a microtiter plate followed by serial 2-fold dilution and the addition of 100 μl of 3% rabbit erythrocytes. The plate was incubated at 37 °C for 1 h and observed for hemolytic activity associated with Act. The supernatants were taken from those wells that showed partial lysis of red blood cells, and the hemoglobin release was recorded at 540 nm by using a $VERSA_{max}$ microplate reader (Sunnyvale, CA).

2.11. Statistics

Wherever applicable, at least three independent experiments were performed. The data were analyzed by using the Student's *t* test, and *P* values of ≤0.05 were considered significant.

3. Results

3.1. DNA methylation by E. coli and A. hydrophila SSU Dam

GidA is a highly conserved protein among different Gram-negative bacteria. The amino acid sequence for GidA from *A. hydrophila* SSU has 98% identity with GidA from *A. hydrophila* ATCC7966^T; 96% identity with GidA of *A. salmonicida* A449; 76% with *Vibrio fischeri*; 75% with *S. enterica*; and 74% identity with the GidA protein of *E. coli* K12 (Blattner et al., 1997; McClelland et al., 2001; Reith et al., 2008; Ruby et al., 2005; Seshadri et al., 2006). The *gidA* gene upstream region of *A. hydrophila* SSU (517-bp) and *E. coli* K12 (381-bp) strains contained 18 and 14 GATC Dam target sites, respectively (Fig. 1). The examination of upstream regions of the *A. hydrophila act* gene indicated the presence of one GATC sequence.

It is known that *Dpn*I enzyme digests Dam-methylated DNA, and *Dpn*II does not digest Dam-methylated DNA. To show that *E. coli* GM28 (Dam$^+$) and *A. hydrophila* SSU (Dam$^+$) strains have identical Dam activity with GATC specificity and that GM33 strain is deficient in the *dam* gene, plasmid DNA (pGlow-Up*gidA*) isolated from *E. coli* strains was treated with *Dpn*I and *Dpn*II restriction endonucleases. The pGlow-Up*gidA* plasmid from the GM28 strain which harbors *dam* was digested with *Dpn*I but not by *Dpn*II (Fig. 2, lanes 2 and 3). The opposite trend was noted when this plasmid DNA isolated from *E. coli* GM33 strain (Δ*dam*) was digested with *Dpn*I and *Dpn*II enzymes (Fig. 2, lanes 4 and 5). Identical digestions were observed for pGlow-Up*act* plasmid DNA (data not shown). *A. hydrophila* gDNA showed a similar digestion pattern with DpnI and DpnII restriction endonucleases (data not shown) as the pGlow-Up*gidA* and pGlow-Up*act* plasmids from *E. coli* GM28 strain, with *Dpn*I and not *Dpn*II digesting gDNA.

3.2. Effect of methylation by Dam on act (Upact) and gidA (UpgidA) expression as a measure of GFP in E. coli dam$^+$ and Δdam genotypes

Table 3 gives the results of the quantitative analysis of relative *act* and *gidA* expressional activities by using gene-encoding GFP as a tran-scriptional reporter in *dam*$^+$ and Δ*dam E. coli* cells. Our data indicated that GFP levels under the control of *act* and *gidA* gene promoters and their upstream sequences which can be methylated from the pGlow-TOPO vector in *E. coli* were dependent on the DNA methylation status. For example, transcriptional activities of Up*act* (91.3±1.7 RFU/mg protein) and Up*gidA* (62.2±1.4 RFU/mg protein) -regions in terms of GFP production were approximately two times lower in the GM28 strain (*dam*$^+$) when compared to that in GM33 strain (Δ*dam*) (183.5 ± 3.1 and 131.7± 1.1 RFU/mg protein, respectively). These results indicated that constitutive methylation by Dam suppressed the activities of both *act* and *gidA* genes as a measure of GFP production in GM28 strain.

3.3. Effect of methylation by Dam on the expression of act (Upact) and gidA (Up*gidA*) in E. coli dam$^+$ ΔgidA and Δdam ΔgidA mutants

To determine the expression of *act* and *gidA* gene upstream regions as a function of GFP production in Δ*gidA* mutants, we deleted the *gidA* gene from the *E. coli* GM28 and GM33 strains. The pGlow-Up*act* and pGlow-Up*gidA* plasmids were transformed in generated *E. coli* mutant strains, and their cell extracts were used for measuring expressional activities, as described above. Table 3 shows no significant differences in the levels of GFP produced through the Up*act* region in *dam*$^+$ Δ*gidA* and Δ*dam* Δ*gidA* mutants (51.7±0.93 versus

52.1±0.21 RFU/mg protein), indicating the role of GidA in modulating Act production regardless of the methylation status of the Up*act*. However, we observed a two-fold difference in the Up*gidA* activity in terms of GFP production between *E. coli* GM28 *dam*$^+$ Δ*gidA* and GM33 Δ*dam* Δ*gidA* strains (60.1±1.0 versus 125.8 ± 1.0 RFU/mg protein), as we observed also for *dam*$^+$ *gidA*$^+$ and Δ*dam gidA*$^+$ backgrounds, indicating the suppressive activity of methylation by Dam on GidA production. This would cause decreased production of Act, as we indeed noted (91.3±1.7 [*dam*$^+$ *gidA*$^+$] versus 183.5 ± 3.1 [Δ*dam gidA*$^+$] RFU/mg protein) (Table 3).

3.4. RT-qPCR analysis

We used RT-qPCR analysis to study whether altered Dam production affected the differential *gidA* and *act* gene expression in parental versus Dam-overproducing *A. hydrophila* SSU strains. The transcriptional expression of *gidA* and *act* genes was 1.9-fold and 2.1-fold higher in Dam-overproducing strain than in the parental strain with constitutive expression of the *dam* gene, respectively. These data verified that *gidA* and *act* gene expression was up-regulated by overproduction of Dam. Additionally, increases in the levels of GFP were observed in *E. coli* Δ*dam* strains when reporter protein GFP was produced from a plasmid under the control of *gidA* (131.7±1.1 RFU/mg protein) or *act* promoter and upstream regions (183.5±3.1 RFU/mg protein) (Table 3). Our results tend to suggest that the hypermethyla-tion of GATC sites upstream of *gidA* and *act* genes in *A. hydrophila* increased Act production, while DNA methylation of these sites by the constitutive production of Dam would have an opposite effect, i.e., reduced Act production.

3.5. Effect of dam and gidA genes on Act-associated hemolytic activity in E. coli

To demonstrate the utility of using *E. coli* K12 as a model to study *act* gene expression of *A. hydrophila* SSU, we tested the hemolytic activity of Act from pBAD-*act* recombinant plasmid in *E. coli* GM28 (*dam*$^+$), GM33 (Δ*dam*) and Δ*gidA* mutants of these strains. The highest hemolytic activity (14.6±0.25) associated with Act (Fig. 3) was noted in the *E. coli* GM28 parental strain (which harbored *dam* and *gidA* genes), which dropped significantly (8.3±0.2) in the Δ*gidA* mutant strain of GM28 in the presence of Dam. As expected, the *E. coli* GM33 mutant strain (Δ*dam* and Δ*gidA*) exhibited hemolytic activity (8.5±0.2) associated with Act similar to that noted for the *E. coli* GM28 Δ*gidA* strain (positive for Dam), further providing evidence of the role of GidA in regulating Act production. We observed a further drop in hemolytic activity (4.3±0.2) of Act in the *E. coli* GM33 strain, which harbored *gidA*, but not the *dam* gene (Fig. 3). *E. coli* strains with pBAD vector alone did not exhibit any hemolytic activity. These data are suggestive that methylation by Dam could vary due to conformation of DNA in the upstream region of the target gene due to the specific genome architecture of specific pathogens.

4. Discussion

The *gidA* gene is conserved among prokaryotes, which indicate that it has a key function within the cell. Indeed, when the *E. coli gidA* mutant was grown on glucose-containing media, it produced long filamentous cells indicating that *gidA* transcription was involved in the initiation of chromosomal replication (Asai et al., 1990; Ogawa and Okazaki, 1994). These results implied that GidA might function to connect glucose metabolism, ribosome function, chromosome replication, and cell division. Recent studies implicated GidA in a number of biological and pathogenic processes, possibly pointing to its global regulatory role. *P. aeruginosa* GidA selectively controlled quorum-sensing gene expression post-transcriptionally via RhlR-dependent and -independent pathways (Gupta et al., 2009). Likewise, *gidA* mutant of *S. pyogenes* produced significantly reduced levels of multiple

virulence factors due to impaired translational efficiencies compared to those of the parental strain (Cho and Caparon, 2008).

The mechanisms that lead to bacterial cell phase variation are classified either as genetic or epigenetic, and Dam belongs to the latter category in that Dam mediates phase variation to control expression and production of some outer surface structures in *E. coli*, e.g., an outer-membrane protein Ag43 (van der Woude, 2008). The methylation state of GATC sites in the *dna*A promoter appeared to be of little consequence to the timing of initiation, as elimination of GATC sites in *dna*A promoter did not result in an initiation timing defect (Wilkinson et al., 2006).

The connection between DNA methylation and promoter activity was found for the herpes simplex virus thymidine kinase gene, when the promoters were methylated in vitro (Levine et al., 1991). These authors showed a possible involvement of protein mediators in the inhibition of promoter activity by methylation associated with a transcriptional inactivity. Well-known *pap* promoter controls the expression of pyelonephritis-associated pili in uropathogenic *E. coli* (UPEC) in response to methylation by Dam (Barnard et al., 2004). Another study demonstrated that the differential methylation by Dam of GATC sequences in the *pap* promoter regulated the expression of pili genes necessary for UPEC cellular adhesion (Peterson and Reich, 2006). These investigators concluded that GATC flanking sequences might be critical for expression of the *pap* promoter and other Dam-regulated genes by affecting the activity of Dam at such sites.

Our earlier published data showed that GidA positively regulated Act production at the translational level and indicated no significant differences in the *act* gene transcription level between the *gidA* mutant and WT strain of *A. hydrophila* (Sha et al., 2004). This observation was confirmed later by Nordman et al. (2007), as *E. coli gidA* transcription levels, which were measured by RNA slot-blot analysis in the *gidA* mutant, were also found to be similar to those seen in WT bacterium.

The GATC Dam methylation sites are unevenly distributed in the genome of *E. coli*, and the *oriC* has a GATC-rich region (Barras and Marinus, 1988). This observation is in agreement with the role of Dam in DNA replication initiation. We noted multiple GATC Dam sites in the upstream region of a *gidA* gene and provided evidence that the expression of the *gidA* gene could be controlled by Dam. It is plausible that Dam might affect the *act* gene directly and/or through the GidA regulator. To test that, we cloned the upstream regions of the *gidA* and *act* genes of *A. hydrophila* into a pGlow-TOPO vector. This promoterless vector gave us the possibility to fuse the promoter region of a gene of interest with the GFP reporter to assay the activity of an upstream region. We observed higher levels of *A. hydrophila* GidA and Act as a function of GFP production in the *dam* mutant of *E. coli* GM33 than in GM28 strain (Table 3). These results are consistent with previous findings about the inverse correlation between methylation and promoter activity showing that the *gidA* transcript level was about 2.5-fold higher in the *dam* mutant compared to that in the *E. coli* PC2 parental strain (Bogan and Helmstetter, 1997). *E. coli* lacking Dam showed an increase in transcription of the genes belonging to the SOS regulon, and a comparison of global gene expression in Δ*dam*, Δ*seqA*, and Dam-overproducing strains showed that the profiles for SeqA-deficient and Dam-overproducing cells were almost identical and distinct from that of Δ*dam* cells (Lobner-Olesen et al., 2003). Similarly, our data provided evidence that levels of GFP produced under the control of *act* and *gidA* gene promoters and upstream sequences were higher in Δ*dam E. coli* compared to that in dam^{+} *E. coli* (Table 3). On the other hand, the *dam* mutant of S. Typhimurium exhibited decreased virulence and also pointed to GATC-binding sites as possibly having a role in regulating the virulence of *Salmonella* and other related bacteria (Chatti and Landoulsi, 2008).

A decreased level of GidA can result in the absence of tRNA modification that may alter the expression of the *act* gene. Therefore, we generated *gidA* deletion mutants of *E. coli* GM28 (*dam*$^{+}$) and *E. coli* GM33 (Δ*dam*) and obtained a double Δ*dam* Δ*gidA* mutant viable strain for the first time for *E. coli* K12. Most interestingly, in *gidA* mutant strains, expression of the *act* gene in the *dam* mutant strain remained at the same low level (52.1±0.21 RFU/mg protein), as in the *dam*$^{+}$ strain (51.7±0.93 RFU/mg protein). Thus, in the absence of the *gidA* gene, Dam does not affect *act* gene expression. However, *gidA* promoter activity, as measured by GFP production in *E. coli* from the plasmid in *gidA* mutant strains, was still dependent on the extent of DNA methylation, showing suppression of the activity in the *dam*$^{+}$ strain (60.1±1.0 RFU/mg protein) compared to the Δ*dam* strain (125.8±1.0 RFU/mg protein) (Table 3). This investigation showed a connection between the amount of Dam produced (constitutive versus overproduction from the plasmid) which modulated the *gidA* gene expression level and then GidA leading to regulation of Act production. Our data suggested that the constitutive expression of the *dam* gene resulted in the decreased expression of both *gidA* and *act* genes in *E. coli* (Table 3), while overproduction of Dam led to an increase in GidA and Act production in *A. hydrophila* (Erova et al., 2006b).

We noted a significant decrease (57%) in hemolytic activity associated with Act in the Δ*gidA* mutant of *E. coli* GM28 (pBAD-*act*) (Fig. 3), which matched with our earlier published data in *A. hydrophila* SSU showing that deletion of the *gidA* gene led to reduced alkaline phosphatase activity associated with the *act::phoA* reporter construct in comparison to the WT strain (Sha et al., 2004). From these results, the contribution of GidA in modulating Act activity was readily evident, and, thus, our data are consistent regarding the modulation of Act by Dam and GidA in *E. coli* versus *A. hydrophila.* Importantly, we observed a further decrease in Act-associated hemolytic activity in the *E. coli Adam gidA*$^{+}$ GM33 strain, compared to *dam*$^{+}$ Δ*gidA*$^{-}$ strain of *E. coli* GM28 (Fig. 3). These data led us to suggest that methylation by Dam of the *gidA* upstream sequence contributes significantly to Act regulation (Fig. 4). These intriguing observations also indicated that methylation caused by *E. coli* Dam of *A. hydrophila gidA* upstream sequence in *E. coli* could be somewhat different when compared to the methylation caused by A *hydrophila* Dam in the homologous strain. Finally, methylation by Dam (hypo- or hyper-) of the *gidA* gene upstream region can affect the expression of the *gidA* gene, which in turn, would regulate the *act* gene in *A. hydrophila* versus *E. coli*.

To investigate the interaction of Dam and GidA on the regulation of *act*, we used RT-qPCR analysis to understand how, in fact, the methylation level of DNA by Dam can affect *gidA* and *act* gene expression. The results indicated that in the *A. hydrophila* SSU Dam-overproducing strain, the transcriptional level of the *gidA* gene was increased two times, but the transcriptional level of the *act* gene also increased two times, which was unexpected, based on our earlier data that GidA modulates Act at the translational level (Sha et al., 2004). Based on the presented data, we can speculate how GidA regulates *act* gene expression. GidA is a global regulatory protein and, thus, modulates the expression of many known and unknown genes, and one of them may affect Act production (Fig. 4). This regulation, by *gidA* for example, can exist at the translational level by tRNA modifications (Gustilo et al., 2008; Saier, 1995).

This study provided evidence that the intermediate protein GidA is involved in the mechanism related to the influence of Dam on bacterial pathogenesis by modulating the activity of the promoter region associated with transcription and possibly indicates that different virulence genes may share a common regulatory mechanism whose activity is dependent on Dam methylation. Specifically for *A. hydrophila*, it appears that once the organism reaches the target site in the human/animal host, Dam is overproduced, which will, in turn, increase Act production, leading to significant tissue damage or diarrhea depending

upon the route of bacterial entry. However, in the natural environmental niche, constitutive levels of Dam will suppress Act production to conserve energy. In the future, we will examine modifying adenine residues of GATC site(s) in the upstream region of the *gidA* gene to show whether this affects Act production in *dam*$^{+}$ and Δ*dam* background strains of *E. coli*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Act	*Aeromonas* cytotoxic enterotoxin
Dam	DNA adenine methyltransferase
tRNA	transfer RNA
GidA	glucose-inhibited division A protein
GATC site	guanine-adenine-thymine-cytosine site
RT-qPCR	real-time quantitative polymerase chain reaction
GFP	green fluorescent protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
DPBS	Dulbecco's phosphate-buffered saline
FAD	Flavin adenine dinucleotide
OriC	origin of replication
Ap	Ampicillin
Cm	Chloramphenicol

Rif	Rifampin
gDNA	genomic DNA
RFU	relative fluorescence units
FRT	flippase [FLP] recognition target
Cm^s	chloramphenicol sensitive
Ap^s	ampicillin sensitive
pap	pyelonephritis-associated pili
***seqA* gene**	sequestration A gene

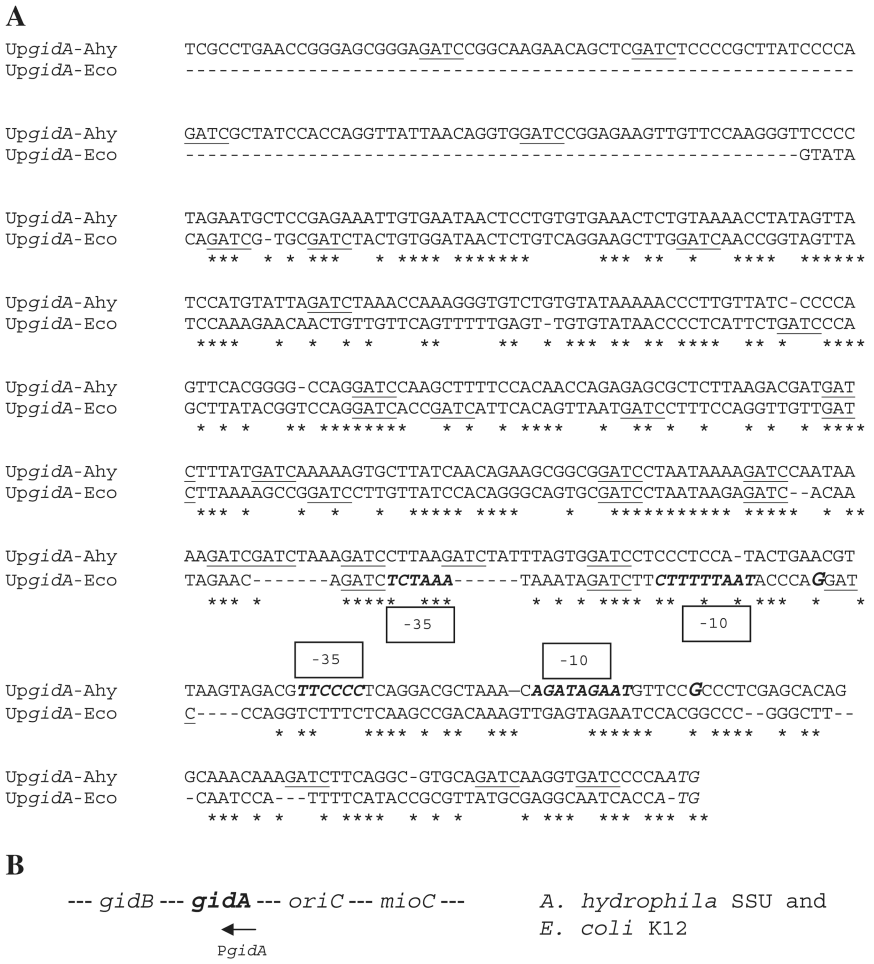

Fig. 1.

A. DNA sequence alignment of *gidA* gene upstream regions from *A. hydrophila* SSU and *E. coli* K12 strains. Asterisks denote conserved nucleotides. The underlined sequences represent GATC sites for Dam methylation. The potential transcriptional start sites (G) and putative -10, -35 boxes are indicated in italics and bold letters. B. Schematic diagram showing *gidA* regions of *A. hydrophila* and *E. coli.* The figure is not drawn to scale.

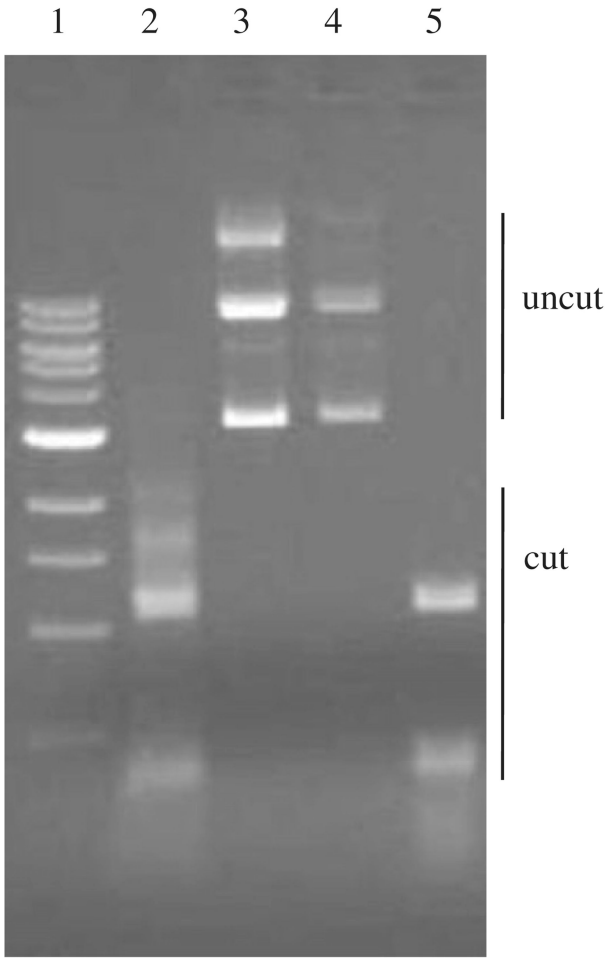

Fig. 2.

*Dpn*I and *Dpn*II cleavage patterns of plasmid DNA isolated from *E. coli* GM28 (*dam*$^{+}$) and GM33 (Δ*dam*) strains containing pGlow-TOPO vector with the upstream region of the *gidA* gene of *A. hydrophila* SSU. Lane 1, 1 kb DNA Ladder (New England BioLabs); lanes 2 and 3, isolated pGlow-Up*gidA* plasmid DNA from *E. coli* GM28 strain treated with *Dpn*I enzyme (lane 2) or *Dpn*II (lane 3); lanes 4 and 5, from *E. coli* GM33 strain containing pGlow-Up*gidA* plasmid digested with *Dpn*I and *Dpn*II, respectively. *A. hydrophila* gDNA showed similar patterns with *Dpn*I and *Dpn*II restriction endonucleases as DNA from *E. coli* GM28 strain (lanes 2 and 3).

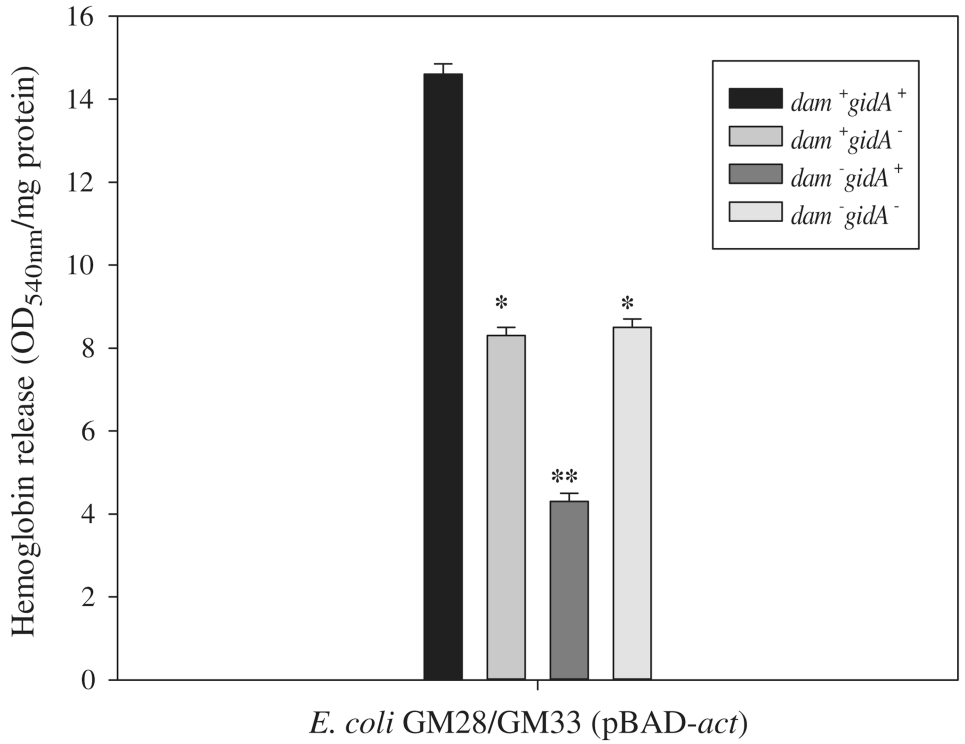

Fig. 3.

Act-associated hemolytic activity in cell extracts of *E. coli* GM28 (pBAD-*act*) and GM33 (pBAD-*act*) in the *gidA*$^+$ and Δ*gidA* background was evaluated by measuring the release of hemoglobin from red blood cells. *E. coli* strains with pBAD vector alone did not exhibit any hemolytic activity (data not shown). * denotes statistically significant difference between *dam*$^+$ *gidA*$^+$ versus *dam*$^+$ *gidA*$^-$ mutants; ** denotes statistically significance between *dam*$^+$ *gidA*$^-$ versus *dam*$^-$ *gidA*$^+$ mutant.

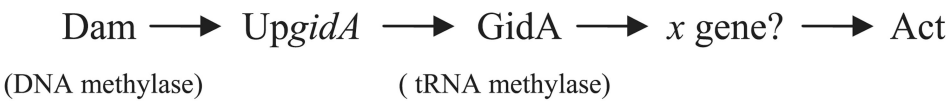

Fig. 4.

A schematic showing the pathway of Act regulation in *A. hydrophila* SSU by Dam and GidA. Dam methylates adenine residue of the GATC sites of the *gidA* upstream sequence (Up*gidA*) to modulate *gidA* expression. GidA with tRNA methylase activity, in turn, possibly through as yet an unknown gene (*x*), leads to Act production.

Table 1

Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)	Source or reference
A. hydrophila SSU-R strains	Rifampin resistance strain, Rif^r	Laboratory
Parental	pBAD plasmid, Rif^r Ap^r	Erova et al. (2006b)
Dam-overproducing	pBAD-*dam* plasmid, Rif^r Ap^r	Erova et al. (2006b)
E. coli K12 strains		
TOP10	F$^-$ *mcr*A Δ *mrr-hsdRMS-mcr*BC Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^r) *end*A1 *nup*G	Invitrogen
GM28	F$^-$ *sup-85* (Am)	Marinus and Morris(1973)
GM33	F$^-$ *dam-3 sup-85* (Am)	Marinus and Morris(1973)
GM28 (pGlow-Up*gidA*)	GM28 with pGlow-Up*gidA* plasmid	This study
GM33 (pGlow-Up*gidA*)	GM33 with pGlow-Up*gidA* plasmid	This study
GM28 (pGlow-Up*act*)	GM28 with pGlow-Up*act* plasmid	This study
GM33 (pGlow-Up*act*)	GM33 with pGlow-Up*act* plasmid	This study
GM28 (pGlow-TOPO)	GM28 with pGlow-TOPO vector	This study
GM33 (pGlow-TOPO)	GM33 with pGlow-TOPO vector	This study
GM28 *gidA* mutant	In-frame *gidA* mutant of GM28	This study
GM33 *gidA* mutant	In-frame *gidA* mutant of GM33	This study
GM28 *gidA* mutant (pGlow-Up*act*)	GM28 *gidA* mutant carrying pGlow-Up*act* plasmid	This study
GM33 *gidA* mutant (pGlow-Up*act*)	GM33 *gidA* mutant carrying pGlow-Up*act* plasmid	This study
GM28 *gidA* mutant (pGlow-Up*gidA*)	GM28 *gidA* mutant carrying pGlow-Up*gidA* plasmid	This study
GM33 *gidA* mutant (pGlow-Up*gidA*)	GM33 *gidA* mutant carrying pGlow-Up*gidA* plasmid	This study
Plasmids		
pGlow-TOPO	Promoterless vector, Ap^r Nm^r	Invitrogen
pGlow-Up*gidA*	pGlow carrying upstream region of *A. hydrophila gidA* gene, Ap^r Nm^r	This study
pGlow-Up*act*	pGlow carrying upstream region of *A. hydrophila act* gene, Ap^r Nm^r	This study
pKD46	λ Red recombinase plasmid, Ap^r	Datsenko and Wanner(2000)
pKD3	*cat* cassette template with FRT sites, Ap^r Cm^r	Datsenko and Wanner(2000)
pCP20	FLP recombinase plasmid, Ap^r Cm^r	Datsenko and Wanner(2000)
pBAD/Thio-E	Prokaryotic expression vector, Ap^r	Invitrogen
pBAD-*act*	*act* gene of *A. hydrophila* cloned into pBAD/Thio-E, Ap^r	This study

Table 2

Primers used in this study.

Primer	Sequence (5′–3′)	Purpose
Up*gidA*-N	TCGCCTGAACCGGGAGCGGGAGATC	PCR amplification of *A. hydrophila* region upstream of *gidA* gene start codon
Up*gidA*-C	ATTGGGGATCACCTTGATCTGCACG	
Up*act*-N	CGGGGTACCGAGCCATGTTATCCCG	PCR amplification of *A. hydrophila* region upstream of *act* gene start codon
Up*act*-C	CCGGAATTCCATAGCAACCCCAATA	
T7-F	TAATACGACTCACTATAGGG	DNA sequencing of the upstream region in pGlow-PCR[a]
GFP-R	GGGTAAGCTTTCCGTATGTA	
F-pKD3	GCCCGGGCTTCAATCCATTTTCATACCGCGTTATG	PCR amplification of a Cm^r cassette for λ Red recombination system
	CGAGGCAATCACCATGTGTAGGCTGGAGCTGCTTC	
R-pKD3	GCGGGTGCTTACCAGGCATTTTTAATGCGTTATGC	
	GCTACGACGCAGCATTATCCTCCTTAGTTCCTATT	
UpF-Cm	TACCCAGGATCCCAGGTCTTTCTCA	PCR verification of *gidA* knockout mutant in GM28 and GM33 *E. coli* strains
UpR-Cm	AGAGGTTCCAACTTTCACCATAATG	
DownF-Cm	ACGCCACATCTTGCGAATATATGTG	
DownR-Cm	AGCGAAATACCTGCGTCTTTCAGCA	
gidA-N	GACATGAACTACCGCGACGTC	RT-qPCR for *gidA* gene
gidA-C	ATGAACCAGCAGGATGGAGATG	
act-N	GCAATATCGAAATCGGTGCGC	RT-qPCR for *act* gene
act-C	GCTGAAGCCAAGCCCGGAGAGC	
gap-1N	AAGCTGACCGGCAACGC	RT-qPCR for *gap*-1 control gene
gap-1C	CAGATAGGCATTGAGGCTCTCC	
*act*N-*Nco*I	CATGCCATGGATGCAAAAACTAAAAATAACTGGCT	Cloning of the *act* gene into pBAD/Thio-E vector
*act*C-*Pme*I	AGCTTTGTTTAAACTTATTGATTGGCTGCTGGCGT	

Underlining indicates FRT and restriction endonuclease sites.

[a] pGlow-Up*gidA* and pGlow-Up*act* plasmids.

Table 3

Level of gene expression as measured by using GFP assay[a] of upstream region of *A. hydrophila act* and *gidA* genes in different Dam background strains of *E. coli*.

Genotype of *E. coli*	Mean activity (RFU/mg protein) ± SD
dam$^+$ *gidA*$^+$ (pGlow-Up*act*)	91.3 ±1.7
Δ*dam gidA*$^+$ (pGlow-Up*act*)	183.5 ± 3.1[b]
dam$^+$ *gidA*$^+$ (pGlow-Up*gidA*)	62.2 ±1.4
Δ*dam gidA*$^+$ (pGlow-Up*gidA*)	131.7±1.1[b]
dam$^+$ Δ*gidA* (pGlow-Up*act*)	51.7 ±0.93
Δ*dam* Δ*gidA* (pGlow-Up*act*)	52.1 ± 0.21
dam$^+$ Δ*gidA* (pGlow-Up*gidA*)	60.1 ±1.0
Δ*dam* Δ*gidA* (pGlow-UpgidA)	125.8 ±1.0[b]

[a] Activity as GFP emission in relative fluorescence unit (RFU) was normalized to the same protein concentration (1 mg) for each indicated *E. coli* strain cell extract.

[b] Statistically significant value ($P \leq 0.05$) using the Student's *t* test.