Biochemical and Biophysical Research Communications xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Vibrio vulnificus AphB is involved in interleukin-8 production via an NF-κB-dependent pathway in human intestinal epithelial cells

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ARTICLE INFO

Article history:
Received 19 December 2011
Available online xxxx

Keywords:
Vibrio vulnificus
AphB
Interleukin-8
Nuclear factor-κB
Intestinal epithelial cell

ABSTRACT

We previously reported that the *aphB* gene mutant of *Vibrio vulnificus* had significantly impaired motility and adherence to host cells. In this study, we investigated the role of *V. vulnificus* AphB on the production of interleukin-8 (IL-8), a proinflammatory cytokine, as well as its underlying mechanism in human intestinal epithelial INT-407 cells. The *aphB* gene mutation significantly reduced the ability of *V. vulnificus* to stimulate IL-8 production and IL-8 gene promoter activation in INT-407 cells. The *V. vulnificus aphB* mutant also induced lower levels of NF-κB DNA binding activity and NF-κB minimal promoter activation than did the wild-type of *V. vulnificus*. Importantly, the observed reductions in IL-8 production, IL-8 gene promoter activation and NF-κB DNA binding activity were significantly restored by complementing the *aphB* gene into the *V. vulnificus aphB* mutant. These results indicate that *V. vulnificus* AphB is involved in the IL-8 production via an NF-κB dependent pathway in human intestinal epithelial cells.

1. Introduction

Vibrio vulnificus is an opportunistic human pathogen that causes septicemia and skin infections, accompanied in many cases by ulcer and edema [1]. This primary septicemia is the most lethal infection caused by *V. vulnificus*, with more than 50% mortality rate. When *V. vulnificus* is ingested orally via contaminated seafood, including shrimp, oysters and clams, it reaches the intestine and invades the bloodstream across the intestinal epithelia of the host [2]. The majority of fatal cases are caused by septic shock, which results from a variety of *V. vulnificus* virulence factors, including hemolysin, capsular polysaccharide, and siderophores [3,4]. These virulence factors may persistently activate the generation of proinflammatory mediators such as IL-1β, IL-8, and nitric oxide in the infected host [5,6].

IL-8, a member of the CXC chemokine family, has been implicated in a variety of infectious diseases. IL-8 is secreted by immune cells and epithelial cells in response to microbial stimuli such as *Helicobacter pylori, Salmonella typhimurium,* and *Vibrio cholera* [7,8]. IL-8 production is regulated by microbial and host factors via transcription factors and cell surface molecules such as Toll-like receptors [9,10]. We previously demonstrated that *V. vulnificus* infection induces IL-8 production in human intestinal epithelial cells by activating NF-κB [6]. Mutation of the NF-κB site within the IL-8 promoter leads to complete non-responsiveness of IL-8 production to *V. vulnificus* infection, thereby underlining the critical importance of NF-κB in IL-8 production.

AphB was initially known as a virulence regulator in *V. cholerae*. *V. cholerae* AphB is a member of the LysR family of transcriptional regulators and plays a central role in virulence gene expression via regulation of the ToxR virulence cascade in response to environmental signals such as oxygen and pH [11]. We previously identified a homologue of *V. cholerae* AphB in *V. vulnificus* with 80% amino acid sequence identity to that of *V. cholerae* Aph [12]. Additionally we demonstrated that the *V. vulnificus aphB* mutant is significantly less virulent than the wild type and is impaired in motility and adherence to host cells [13]. However, the role of AphB in proinflammatory responses of host cells remains to be elucidated.

In this study we investigated the roles of *V. vulnificus* AphB in IL-8 production as well as its underlying mechanisms in human intestinal epithelial cells.

2. Materials and methods

2.1. Bacterial strains, cell culture and reagents

V. vulnificus strain ATCC 29307 used in this study was obtained from the ATCC (Manassas, VA, USA), and the *aphB* gene mutant and complemented strains of *V. vulnificus* were constructed, as previously described [13]. For the infection experiments, the bacteria

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0006-291X/$ - see front matter © 2012 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2011.12.122

were grown overnight at 30 °C in Luria-Bertani (LB) medium supplemented with 2.0% NaCl (LBS medium), and diluted to 6×10^8 CFU/ml in LBS, then centrifuged and resuspended in antibiotic-free growth medium prior to infection into INT-407 cells. The INT-407 cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) (growth medium) in an atmosphere of 5% CO_2 at 37 °C. The *V. vulnificus* flagella were prepared and purified, as previously described [14]. Purified flagella were identified by western blotting using a polyclonal *V. vulnificus* flagellin B antibody. The concentration of the LPS in the purified fraction was less than 0.05 endotoxin units in 10 μg/ml of protein, as determined using the Limulus Amebocyte lysate QCL-1000 kit (BioWhittaker, Walkersville, MD).

2.2. Infection protocol

Human intestinal INT-407 cells were infected with *V. vulnificus*, as previously described [15]. Briefly INT-407 cells were grown in culture flasks at 37 °C in a 5% CO_2 incubator. The cells were seeded onto each well of six-well culture plates (5×10^5 cells/well) and cultured for 24 h in antibiotic-free growth medium. Prior to infection, the bacteria were centrifuged for 3 min at 5000 rpm, resuspended, and adjusted to 6×10^8 CFU/ml in antibiotic-free MEM. The bacterial suspensions were added to epithelial cells at a multiplicity of infection (MOI, ratio of bacteria no. to epithelial cell no.) from 1 to 10, after which the infected cells were incubated in a 5% CO_2 incubator for various durations at 37 °C in antibiotic-free growth medium.

2.3. IL-8 ELISA

INT-407 cells were infected with *V. vulnificus* at various MOIs or various incubation times, after which the cells were washed in PBS and post-cultured for 18 h in MEM medium containing gentamicin (100 μg/ml). The culture supernatants were then analyzed for the levels of IL-8 protein using the OptEIA™ human IL-8 ELISA kit 2 (BD Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions.

2.4. RT-PCR analysis

Total RNA was isolated from the cells and reverse-transcribed into cDNA, and then PCR amplification of the cDNA was performed. The primers used were as follows: human IL-8 (302 bp), 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ (sense) and 5′-TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC-3′ (antisense); β-actin (373 bp), 5′-TTCTACAATGAGCTGCGTGTGGCT-3′ (sense) and 5′-GCTTCTCCTTAATGTCACGCACGA-3′ (antisense). Reactions were conducted in an MJ Thermal Cycler (Watertowm, MA, USA) for 36 cycles of the following: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extending at 72 °C for 30 s.

2.5. Transient transfection and luciferase reporter assay

INT-407 cells were transiently transfected with human IL-8 gene promoter, or with NF-κB minimal promoter constructs, as previously described [6]. Some cells were transfected with IL-8 gene promoter containing a mutation of NF-κB site. A linker-scanning mutation was generated by a two-step polymerase chain reaction procedure with overlapping internal primers that contain mutated sequences for the NF-κB site. For transfections, INT-407 cells were transiently transfected with each of the indicated promoter constructs by using Superfect method (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The transfected cells were incubated for 12 h at 37 °C in a 5% CO_2 incubator, followed by infection with *V. vulnificus* for 1 h in antibiotic-free MEM at various MOIs. The cells were washed in PBS, after which the cells were incubated for 18 h in the presence of gentamicin (100 μg/ml). Afterwards, the cells were harvested and suspended for 20 min in 50 μl of lysis buffer. The supernatant fluid was harvested and assayed for luciferase activity using Luciferase Reagent (Promega) in a Luminometer. The results were normalized to *LacZ* expression, and were expressed as relative fold induction.

2.6. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

INT-407 cells (8×10^5 cells/dish) were seeded onto 60 mm culture dishes and cultured for 24 h in antibiotic-free growth medium, and then infected for 1 h with *V. vulinficus* at various MOIs. The nuclear extracts from the INT-407 cells were prepared and the EMSA was performed as previously described [6]. An oligonucleotide harboring an NF-κB binding site (5′-CCGGTTAACAGAGGGGGCTTTCCGAG-3′) was used as a probe. Specific binding was verified via competition experiments with a 50-fold excess of unlabeled, identical oligonucleotides of cAMP response element (CRE)-containing oligonucleotides.

2.7. Statistical analyses

The Student's *t*-tests and one-way analysis of variance (ANOVA), followed by the Bonferroni method, were used to determine the statistical differences between the values of the various experimental and control groups. A *p*-value less than 0.05 was considered to be statistically significant.

3. Results

3.1. The aphB gene mutant of V. vulnificus induces significantly lower levels of IL-8 production in human intestinal epithelial cells than the wild-type

To investigate the role of the *V. vulnificus aphB* gene in the induction of IL-8 production in human epithelial cells, human intestinal epithelial INT-407 cells were infected with the *aphB* gene mutant strain or the wild-type strain of *V. vulnificus* as a control, and the levels of IL-8 expression were determined.

As shown in Fig. 1A, infection with the wild-type *V. vulnificus* profoundly induced IL-8 production by INT-407 cells, and the levels were higher than those of LPS or flagella treatment (205.8 ± 40.5 and 165.2 ± 23.4 pg/ml, respectively). Infection with the *V. vulnifcus aphB* mutant induced significantly lower levels of IL-8 production in human intestinal epithelial INT-407 cells than did the wild-type at various MOIs and treatment times. The INT-407 cells exposed to the *aphB* mutant for 1 h at an MOI of 10 secreted 170.8 ± 29.4 pg/ml of IL-8 for 18 h, significantly lower than the levels (318.8 ± 26.4 pg/ml) secreted by the wild type-stimulated INT-407 cells. Heat-killed *V. vulnificus* decreased the inductive effects of *V. vulnificus* on IL-8 production and, however, the lowering effects of IL-8 production by the *aphB* mutation were still observed (Fig. 1B).

Furthermore, to determine whether the decreased IL-8 production by the *aphB* mutant is the results of decreased IL-8 mRNA expression, the effects of the *aphB* gene mutation on IL-8 mRNA expression were determined in INT-407 cells infected with the *aphB* mutant or the wild-type. As shown in Fig. 1C, the levels of IL-8 mRNA were significantly lower in the intestinal epithelial cells with the *aphB* mutant than the wild type. In contrast, neither the *aphB* mutant nor the wild-type affected β-actin mRNA expression, thereby indicating that the effects of *aphB* gene mutation on IL-8

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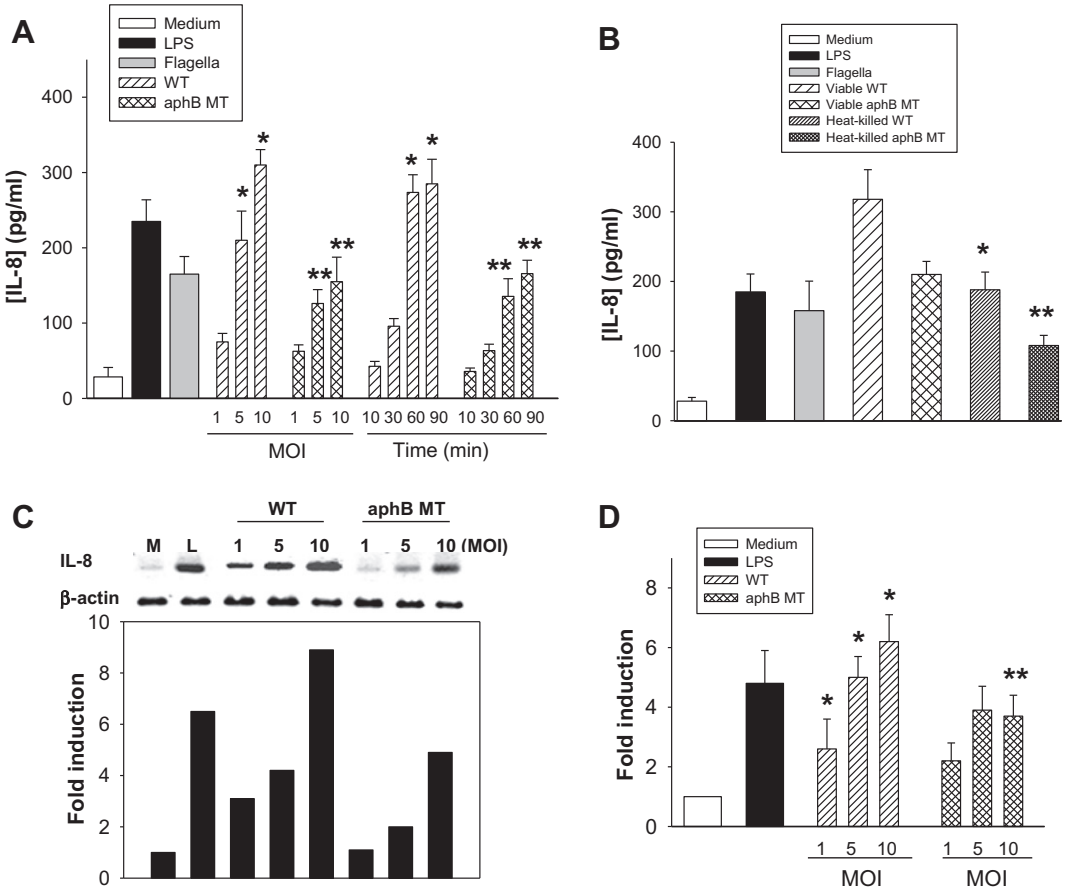

Fig. 1. The *aphB* gene mutation reduces the ability of *V. vulnificus* to stimulate IL-8 production and IL-8 promoter activity in INT-407 cells. (A) INT-407 cells were infected with the wild-type (WT) or the *aphB* mutant-type (MT) at various MOIs and treatment times (MOI = 5). As controls, INT-407 cells were incubated with 10 μg/ml LPS, 10 μg/ml purified flagella, or medium alone. Then, the cells were washed with PBS and further incubated for 18 h, after which IL-8 levels were analyzed by ELISA. Data represent the means ± SD ($n = 3$). $^{*}p < 0.001$, *vs.* a group of medium alone. $^{**}p < 0.05$, *vs.* a group of WT. (B) INT-407 cells were infected for 1 h with viable or heat-killed WT or *aphB* MT at an MOI of 10. Data represent the means ± SD ($n = 3$). $^{*}p < 0.05$, *vs.* a group of treatment with viable WT. $^{**}p < 0.05$, *vs.* a group of heat-killed aphB MT. (C) INT-407 cells were infected for 30 min with WT or *aphB* MT at various MOIs and IL-8 mRNA expression was determined. Band intensity is represented as relative fold to the control treated with medium alone. Data are representative of three independent experiments. (D) INT-407 cells were transfected with IL-8 gene promoter, followed by 1 h of infection with WT or *aphB* MT at an MOI of 10. Afterwards, the luciferase activity was determined. The results are expressed as the induction fold over the value of the uninfected INT-407 cells transfected with the IL-8 promoter. Data represent the means ± SD ($n = 3$). $^{*}p < 0.05$, *vs.* a group of medium alone. $^{**}p < 0.05$, *vs.* a group of WT.

expression were not the result of a general dampening of cellular activation.

To determine whether the *aphB* gene mutation in *V. vulnificus* also affected IL-8 gene promoter activity, INT-407 cells were transfected with the IL-8 gene promoter/luciferase construct, and were then infected with either the *aphB* mutant or the wild-type of *V. vulnificus*, after which the luciferase activity was assessed. As indicated (Fig. 1D), the IL-8 promoter construct showed significant stimulation with *V. vulnificus* infection in an MOI-dependent manner. However, the stimulated levels were significantly lower in the INT-407 cells infected with the *aphB* mutant than in those infected with the wild-type. These results indicate that the decreased IL-8 induction by the *aphB* mutant occurred at the mRNA level.

3.2. The V. vulnificus aphB gene mutant induces lower levels of NF-κB DNA binding activity

We previously reported that the NF-κB binding site was required to optimally express the IL-8 gene after *V. vulnificus* infection [6]. To determine whether NF-κB activity is involved in the reduced levels of IL-8 production in human intestinal epithelial cells infected with the *V. vulnificus aphB* mutant, the NF-κB DNA binding activity were analyzed in the nuclear extract of the wild-type- and the *aphB* mutant-infected INT-407 cells. As shown in Fig. 2A, the nuclear extracts from the wild type *V. vulnificus*-infected INT-407 cells exhibited strong NF-κB binding activity, as expected. In contrast, the *aphB* mutant induced significantly lower levels of NF-κB DNA binding activity than did the wild-type, as demonstrated by the electrophoretic mobility shift assay (EMSA) using a labeled oligonucleotide containing a consensus NF-κB binding site. This binding was specific, as it competed with an unlabeled, identical oligonucleotide, but not with an unrelated, non-specific oligonucleotide, and was absent in the nuclear extracts from the non-infected cells.

To further investigate whether NF-κB activity was involved in the reduced levels of IL-8 production in human epithelial cells infected with the *V. vulnificus aphB* mutant, INT-407 cells were transiently transfected with the NF-κB minimal promoter/luciferase construct, followed by *V. vulnificus* infection, and the luciferase activity was determined. As shown in Fig. 2B, the NF-κB minimal promoter construct showed potent stimulation with the wild-type *V. vulnificus* in an MOI-dependent manner. However, the levels of NF-κB transactivation decreased significantly in INT-407 cells infected with the *V. vulnficus aphB* mutant. Next, we introduced a linker scanning mutation into the NF-κB binding site within the −144/+44 construct (IL-8/LS promoter, Fig. 2C) to directly test

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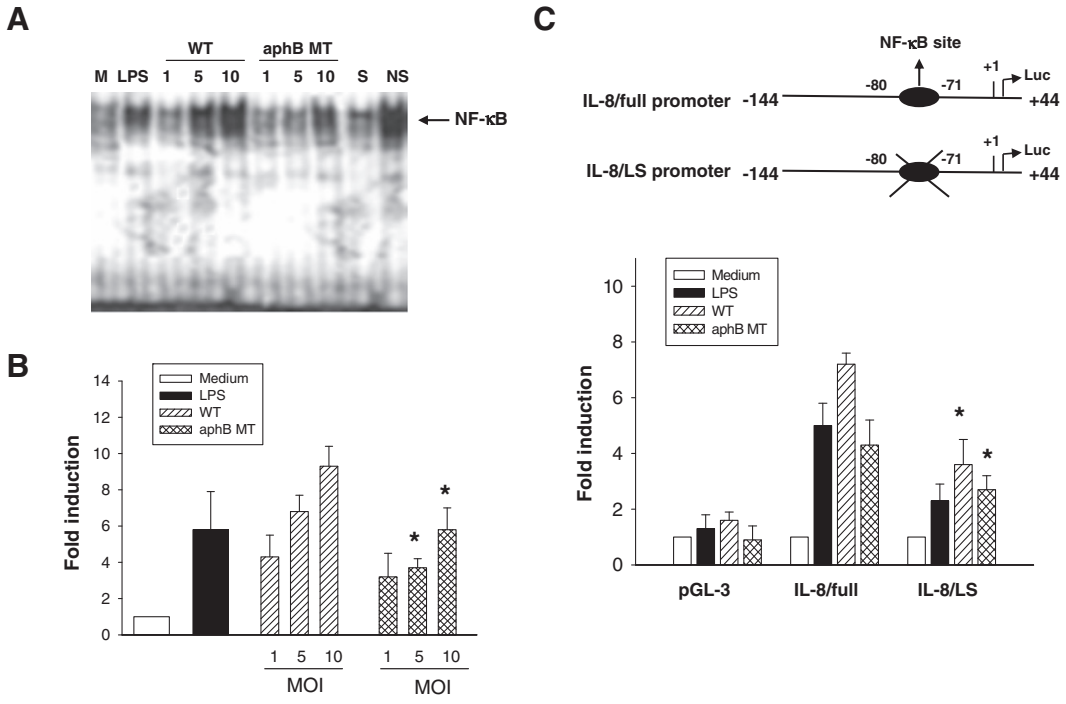

Fig. 2. The *V. vulnificus aphB* mutant induces lower levels of NF-κB activation in INT-407 cells than does the wild-type. (A) INT-407 cells were infected for 1 h with the WT or *aphB* MT at various MOIs, or stimulated with 10 μg/ml LPS. The nuclear extracts were examined for NF-κB DNA binding activity. S and NS indicate the presence of a 50-fold excess of specific oligonucleotide (NF-κB) and non-specific oligonucleotide (CRE), respectively. Data are representative of three independent experiments. (B) INT-407 cells were transfected with NF-κB minimal promoter, followed by 1 h of infection with the wild-type or *aphB* MT at various MOIs. Then, the luciferase activity was determined. The results are expressed as the induction fold over the value of the uninfected INT-407 cells transfected with NF-κB minimal promoter. Data represent the means ± SD ($n = 3$). $^*p < 0.05$, *vs.* a group of WT. (C) INT-407 cells were transfected with the unmodified pGL-3 vector, IL-8 full promoter, or NF-κB binding site-mutated IL-8 promoter (IL-8/LS promoter), followed by 1 h of infection with the wild-type or *wbpP* mutant of *V. vulnificus* at an MOI of 10. Afterwards, the luciferase activity was determined. The results are expressed as the induction fold over the value of the uninfected INT-407 cells transfected with each of the promoter constructs. Data are the means ± SD ($n = 4$). $^*p < 0.05$, *vs.* a group of IL-8 full promoter.

the role of the NF-κB binding site found between −80 and −71 of the IL-8 full promoter. As shown in Fig. 2C, *V. vulnificus aphB* gene-dependent promoter activation was still observed with the IL-8/LS promoter, although it was significantly reduced compared with IL-8/full promoter. However, the decreased effect of the *aphB* mutant on IL-8 promoter activation was not observed in the IL-8/LS promoter, in comparison to the IL-8/full promoter, clearly indicating that the stimulatory effect of *V. vulnificus aphB* gene on IL-8 production might be mediated through the κB binding site. These data indicate that the inhibitory effect of the *V. vulnificus aphB* gene mutation on IL-8 production was mediated via down-regulation of NF-κB activity.

3.3. Complementation of the aphB gene in the aphB mutant restored the decreased IL-8 production and NF-κB activity

To determine whether the complementation of the *aphB* gene into the *V. vulnificus aphB* mutant ameliorates the reduction in IL-8 production induced by the *aphB* mutant, INT-407 cells were infected with the wild-type, *aphB* mutant, and *aphB*-complemented *V. vulnificus* strains, and the levels of IL-8 production were determined. As shown in Fig. 3A, the *aphB* mutant induced significantly lower levels of IL-8 production in INT-407 cells than did the wild-type, and these levels were restored as a result of *aphB* gene complementation in the *V. vulnificus aphB* mutant. Additionally, IL-8 mRNA levels and IL-8 promoter activity were restored in INT-407 cells infected with *aphB*-complemented *V. vulnificus* (Fig. 3B and C).

To further determine whether the complementation of the *aphB* gene in the *V. vulnificus aphB* mutant would restore the decreased NF-κB activity by the *V. vulnificus aphB* mutant, both EMSA and NF-κB minimal promoter assay were conducted in INT-407 cells infected with the wild-type, *aphB* mutant, and *aphB*-complemented *V. vulnificus*. As shown in Fig. 4, the *aphB* mutant induced lower levels of NF-κB DNA binding activity in INT-407 cells than did the wild-type, which was partially restored by complementation of the *aphB* gene in the *aphB* mutant. Additionally, the reduced NF-κB minimal promoter activity by the *aphB* gene mutation was restored by complementation of the *aphB* gene (Fig. 4B).

4. Discussion

We demonstrated for the first time that the *V. vulnificus aphB* gene was involved in the induction of IL-8 production in human intestinal epithelial cells. The *aphB*-mediated IL-8 production might occur via NF-κB activation. As IL-8 is a chemokine that plays a primary role in the mobilization of cellular defense mechanisms [16] and may signal the onset of an acute inflammatory response, the *V. vulnificus aphB* gene may constitute an important mediator of inflammatory responses, which contributes to the epithelial injury inherent to a *V. vulnificus* infection. We reported previously that the *aphB* gene mutation in *V. vulnificus* led to impaired ability to attach to the epithelial cells *in vitro*, thereby resulting in an attenuation of virulence in mice [13]. Many other studies have reported that infecting intestinal epithelial cells with pathogenic bacteria such as *Bacillus subtilis*, *H. pylori*, or *S. typhimurium*, resulted in increased IL-8 production [17,18].

The mechanism by which the *aphB* mutant induced significantly lower levels of IL-8 production at the molecular level remains uncertain. In our study, the *aphB* gene mutation in *V. vulnificus*

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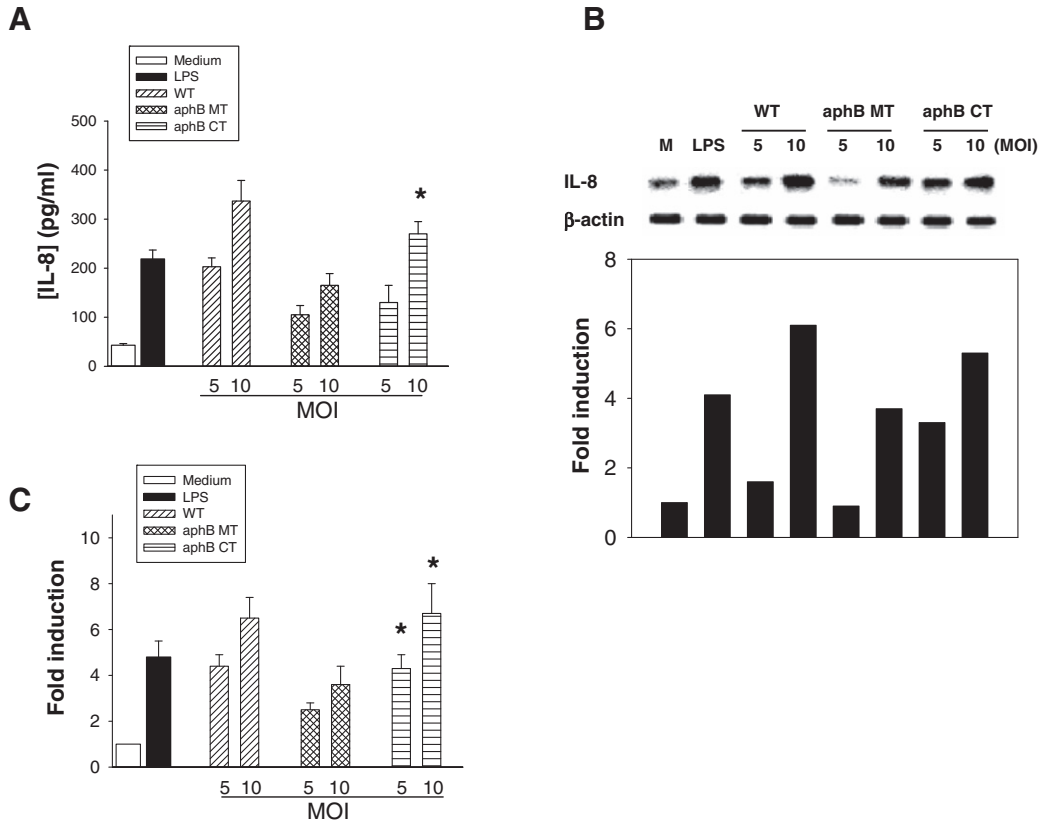

Fig. 3. Complementation of *aphB* gene into the *V. vulnificus aphB* mutant restores the decreased IL-8 production in INT-407 cells. (A) INT-407 cells were infected for 1 h with WT, *aphB* MT, and *aphB*-complemented *V. vunificus* (*aphB* CT), followed by washing with PBS and further incubation for 18 h, after which IL-8 levels were analyzed. $^{*}p < 0.05$, *vs.* a group of *aphB* MT. (B) INT-407 cells were infected for 1 h with *V. vunificus* WT, *aphB* MT, and *aphB* CT, and IL-8 mRNA expression was determined via RT-PCR. Band intensity was expressed as relative fold to the control treated with medium alone. Data are representative of three independent experiments. (C) INT-407 cells were transfected with IL-8 promoter, followed by 1 h of infection with WT, *aphB* MT, and *aphB* CT of *V. vunificus*. Then, the luciferase activity was determined. The results are expressed as the induction fold over the value of the uninfected INT-407 cells transfected with IL-8 promoter. Data are the means ± SD ($n = 4$). $^{*}p < 0.05$, *vs.* a group of *aphB* MT.

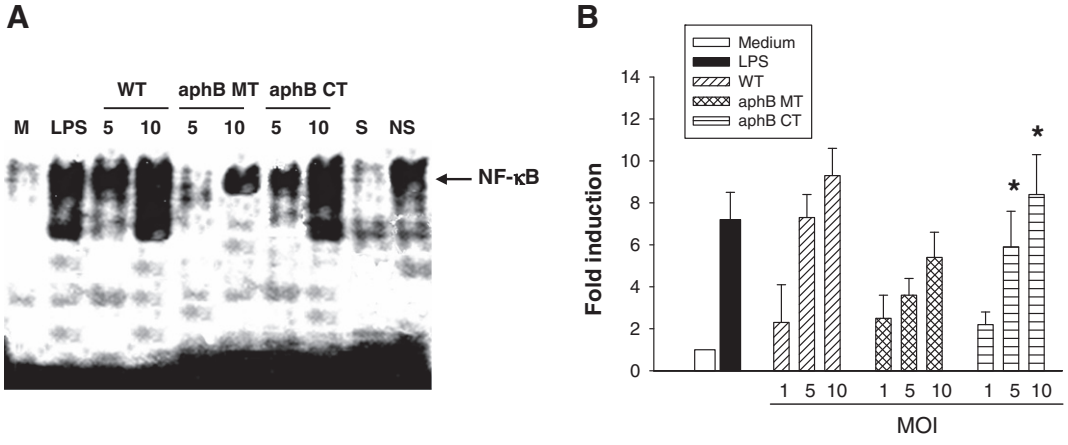

Fig. 4. Complementation of the *aphB* gene into the *aphB* mutant restores the NF-κB activation activity decreased by the *aphB* gene mutation. (A) INT-407 cells were infected for 1 h with WT, *aphB* MT, and *aphB* CT, after which the NF-κB DNA binding activity were determined. Data are representative of three independent experiments. (B) INT-407 cells were transfected with NF-κB minimal promoter, followed by 1 h of infection with WT, *aphB* MT, and *aphB* CT, after which the luciferase activity was determined. The results are expressed as the induction fold over the value of the uninfected INT-407 cells transfected with NF-κB minimal promoter. Data are the means ± SD ($n = 4$). $^{*}p < 0.05$, *vs.* a group of *aphB* MT.

attenuated NF-κB promoter activity in response to *V. vulnificus* infection (Fig. 2B), which was restored by complementation of the *aphB* gene into the *aphB* mutant (Fig. 4B), indicating that the reduced levels of IL-8 production by the *aphB* mutant may be mediated via NF-κB. Furthermore, infecting INT-407 cells with the wild-type *V. vulnificus* resulted in marked enhancement of DNA binding activity to the NF-κB sites, which were strongly decreased when INT-407 cells were infected with the *aphB* mutant *V. vulnificus* (Fig. 2A). The NF-κB transcription factor plays a role in the inducible transcription of the IL-8 gene in mammalian cells, as

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the promoters of both human and murine IL-8 genes harbor NF-κB binding sites [12].

The decreased IL-8 production in *V. vulnificus aphB* mutant-infected intestinal cells may result from the impaired adherence of the *aphB* mutant to intestinal cells or defective expression of other virulence factors from the *aphB* mutant. The *V. vulnificus aphB* mutant has a decreased ability to attach to human intestinal cells [13]. *V. vulnificus* FlaB directly binds to human TLR5 expressed on cultured epithelial cells, leading to the activation of NF-κB and IL-8 production [19]. However, as *V. cholerae aphB* is involved in the expression of several virulence factors [11], further studies should be performed to identify and compare the secreted molecules from the wild type and the *aphB* mutant of *V. vulnificus* in the absence or presence of host cells. Furthermore, in addition to the *aphB* gene-regulated factors, other *V. vulnificus* genes and molecules may also be involved in the induction of IL-8 production in human epithelial cells. The *aphB* gene mutation of *V. vulnificus* did not result in reduced levels of IL-8 expression and NF-κB activity to the baseline of the uninfected cells (Figs. 1 and 2). The *aphB* mutant still induced substantial levels of IL-8 expression in human epithelial cells, although these levels remained significantly lower than those induced by the wild-type. Mutations of *rtxE* and *wbpP* genes in *V. vulnificus* have been reported to reduce IL-8 production in human epithelial cells [15,20].

In conclusion, we have demonstrated that the *aphB* mutant of *V. vulnificus* induced significantly lower levels of IL-8 production than the wild-type, and that NF-κB was involved in the reduced IL-8 production caused by the *aphB* gene mutation. These results indicate that the *aphB* gene-mediated molecules may play an important role as a virulence factor to induce proinflammatory cytokines such as IL-8 in human intestinal epithelia cells.

Acknowledgments

This work was supported by Grants from the National Research Laboratory [R0A-2007-000-20039-0] through National Research Foundation; and the Agriculture Research Center Program of the Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

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