J. Microbiol. Biotechnol. (2006), **16**(9), 1416–1421

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation of *N*-Acetylmuramoyl-L-Alanine Amidase Gene (*amiB*) from *Vibrio anguillarum* and the Effect of *amiB* Gene Deletion on Stress Responses

AHN, SUN-HEE, DONG-GYUN KIM, SEUNG-HA JEONG, GYEONG-EUN HONG, AND IN-SOO KONG*

Department of Biotechnology and Bioengineering, Pukyong National University, Busan 608-737, Korea

Received: March 8, 2006
Accepted: April 6, 2006

Abstract We identified a gene encoding the *N*-acetylmuramoyl-L-alanine amidase (*amiB*) of *Vibrio anguillarum*, which catalyzes the degradation of peptidoglycan in bacteria. The entire open reading frame (ORF) of the *amiB* gene was composed of 1,722 nucleotides and 573 amino acids. The deduced amino acid sequence of AmiB showed a modular structure with two main domains; an N-terminal region exhibiting an Ami domain and three highly conserved, continuously repeating LysM domains in the C-terminal portion. An *amiB* mutant was constructed by homologous recombination to study the biochemical function of the AmiB protein in *V. anguillarum*. Transmission electron microscopy (TEM) revealed morphological differences, and that the mutant strain formed trimeric and tetrameric unseparated cells, suggesting that this enzyme is involved in the separation of daughter cells after cell division. Furthermore, inactivation of the *amiB* gene resulted in a marked increase of sensitivity to oxidative stress and organic acids.

Key words: *N*-Acetylmuramoyl-L-alanine amidase, *amiB* gene, *Vibrio anguillarum*

Vibrio anguillarum, a Gram-negative aquatic bacterium, is highly pathogenic towards different species of marine fish. It is the main causative agent of vibriosis, and this disease results in a lethal hemorrhagic septicemia that causes great economic damage to the aquaculture industry. Similar to most virulence bacteria that produce a variety of virulence factors, *V. anguillarum* also secretes a number of possible pathogenic factors including metalloprotease, lipopolysaccharide, hemolysin, hemagglutinin, and siderophore implicated in the iron-sequestering system [25]. Although genetic studies on the iron uptake system and a transmembrane regulatory protein of *V. anguillarum* have been well characterized [5], many detailed informations are still lacking.

Peptidoglycan, a major component of bacterial cell wall, is essential for the maintenance of cell integrity. The basic structure of peptidoglycan form a network by (β 1-4)-glycosidic bonds of glycan chains and peptide bonds linked to the glycan. It is important for preservation of cell shape and protection of osmotic lysis. Peptidoglycan is degraded by actions of several bacterial hydrolases, which can break specifically linkage bonds in their own cell wall peptidoglycan. Peptidoglycan hydrolases, also called autolysins, are synthesized during cellular growth and are involved in various cellular functions that require cell wall turnover, cell expansion, separation, lysis motility, competence and protein secretion [6, 7, 18, 21, 22, 24, 26]. According to different cleavage specificities, autolysins are classified as *N*-acetylmuramidase, *N*-acetylglucosaminidase, *N*-acetylmuramoyl-L-alanine amidase, and endopeptidase.

Autolysins produced by pathogenic Gram-positive bacteria are known to contribute to the pathogenicity. The autolysin (Ami) of *Listeria monocytogenes* plays a role in its adhesion to eukaryotic cells, leading to inflammation by generating cell-wall degradation products, and its colonization in the liver of mice [14, 17]. In *Streptococcus pneumoniae*, the autolytic function of wild strain is necessary for virulence, and an autolysin-defective mutant exhibits attenuated virulence in a rat model [1, 15]. Recently, it has also been reported that an autolytic protein (AtlA) identified from *S. mutants* plays a very important role in biofilm formation to adhere to host tissues [4, 20]. Although autolysins are believed to play a pivotal function in cell metabolism and pathogenicity of Gram-positive bacteria, only a limited number of autolysins from Gram-negative bacteria have extensively been investigated. In this study, we cloned and analyzed the *N*-acetylmuramoyl-L-alanine-amidase (*amiB*) gene of *V. anguillarum*. We then constructed an *amiB* mutant by the allelic exchange method. Comparison of electron microscopic observation led us to suggest that the AmiB

*Corresponding author
Phone: 82-51-620-6185; Fax: 82-51-620-6180;
E-mail: iskong@pknu.ac.kr

protein of *V. anguillarum* may be required for cell separation. The effect of *amiB* mutation on survival in the presence of organic acids and hydrogen peroxide was examined.

MATERIALS AND METHODS

Bacterial Strains and Culture Condition

The bacterial strains and plasmids used in this study are listed in Table 1. Brain-heart infusion (BHI; Difco Laboratories) and Luria-Bertani (LB; Difco Laboratories) broth and agar were used to grow *V. anguillarum* and *Escherichia coli* strains, respectively. Antibiotics were used at the following concentrations: 100 μg of ampicillin per ml for *E. coli* and 15 μg of chloramphenicol per ml for the *amiB* mutant.

Cloning of *amiB* Gene from *V. anguillarum*

Procedures for DNA, such as DNA isolation, endonuclease restriction, ligation, and agarose gel electrophoresis, were performed as described by Sambrook and Russell [23]. The cloning vector pGEM-4Z was used to generate a genomic DNA library from *V. anguillarum*. For sequence analysis of the insert DNA fragment, each clone was analyzed using a PRISM 377 automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.) with a designed primer from the SP6 promoter region. One clone, containing a 5-kb insert, had an *N*-acetylmuramoyl-L-alanine amidase gene and was named pAMI5. Sequence similarity analysis was done using the BLAST and FASTA network server of the National Center for Biotechnology Information [3, 10].

Construction of *amiB* Knockout Mutant

To construct the *V. anguillarum* strain deficient in the production of AmiB, a 0.6-kb *amiB* fragment (991-1587) was amplified by polymerase chain reaction (PCR) from *V. anguillarum* chromosomal DNA using the primers 5'-GGCCGTCGACGAGGGTTACAAGGTGGCAACT-3' and 5'-GGCCGAGCTCCCCAACGTTAAGGTGTCTGAA-3', which contained SalI and SacI sites, respectively (underlined). The resulting fragment was isolated from an agarose gel and ligated into the allelic exchange suicide vector pNQ705 [13], which had been linearized with SalI and SacI. The resulting plasmid pAMI6 was introduced into the conjugal donor *E. coli* SM10λ*pir*. Conjugation was carried out between the recipient *V. anguillarum* and the donor *E. coli* strain containing pAMI6. A conjugant carrying a single-crossover mutation of *amiB* was obtained by selection on thiosulfate citrate bile salts (TCBS) agar containing 15 μg/ml chloramphenicol, and was confirmed to have an insertion into the *amiB* gene by PCR analysis.

Susceptibility of *V. anguillarum* and *amiB* Mutant to Oxidative Stress

Cells grown in BHI medium to an early stationary growth phase (optical density of 1.2 at 600 nm) were harvested by centrifugation and the pellet was resuspended in PBS. One-hundred mM H_2O_2 was added to 100 μl of the cell suspension and incubated at room temperature. Samples were collected every 10 min for 50 min after the addition of H_2O_2. At indicated time points, appropriate bacterial dilutions after serially diluting were plated onto BHI agar plates. The survival was calculated by dividing the number of CFU at different time points by the initial number of CFU at time zero. Experiments were performed at least three times with duplicate samples.

Tolerance to Organic Acid

To determine the resistance of *V. anguillarum* and *amiB* mutant to organic acid (lactate and acetate, respectively), cells were cultured as described above. Lactate and acetate were added to cell cultures, and then incubated at 25°C for 3 h (concentration of organic acid ranged from 0 mM to

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics[a]	Reference or source
Vibrio anguillarum		
NB10	Serotype O1	[11]
amiB mutant		This study
Escherichia coli		
DH5α	*SupE44 ΔlacU169* (Φ80 *lacZ* ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen Co.
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*; Km^r; host for for π-requiring plasmids; conjugal donor	[12]
Plasmids		
pGEM-4Z	Cloning vector; Ap^r	Promega Co.
pNQ705	Cloning vector; R6K γ *ori* (requires π); *oriT* of RP4; Cm^r	[12]
pAMI5	pGEM-4Z with 5-kb BamHI fragment containing *amiB* from *V. anguillarum*	This study
pAMI6	pNQ705 with 0.6-kb SalI-SacI fragment internal coding sequence of *amiB*; for allelic exchange	This study

[a]Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamysin resistant.

2 mM lactate, 0 mM to 1 mM acetate, respectively). Acid shock cultures were harvested by centrifugation and the pellet was resuspended in PBS. Suspensions were pelleted and the supernatants were removed. The cell pellets were resuspended in saline, and viable cells were assessed by CFU on BHI agar plates as described above.

Nucleotide Sequence Accession Number

The 1,722-bp nucleotide sequence determined in this study was deposited in GenBank under accession number DQ431190.

RESULTS

Cloning and Sequencing of *amiB* Gene from *V. anguillarum*

One 5-kb BamHI fragment containing an amidase gene was cloned from a *V. anguillarum* DNA library and the plasmid DNA, named pAMI5, was isolated. Translation of the nucleotide sequence revealed an ORF of 1,722 bp, encoding a polypeptide of 573 amino acid residues, with a calculated molecular mass of 62,920 Da. The proposed ATG translation start codon was preceded by a probable ribosome binding site, AAGG. An inverted repeat, which could function as a transcription termination signal, was found 48 bp downstream from the stop codon.

Alignment of the deduced *N*-acetylmuramoyl-L-alanine amidase amino acid sequence with four *amiB* homologs from *V. cholerae* (ZP00748124), *V. parahaemolyticus* (BAC61083), *V. vulnificus* (BAC95838), and *V. fischeri* (YP205709) showed 77, 66, 65, and 66% identities, respectively. The deduced amino acid sequence of AmiB showed a modular structure with two main domains: an N-terminal region exhibiting the Ami domain and a C-terminal portion containing three repeated LysM motifs. These conserved domains are common to sequences of vibrio autolysins.

Physiological Characterization of *amiB* Mutant

After insertional disruption of the *amiB* gene was derived by the homologous recombination method with a suicide vector, pNQ705, we examined for physiological differences between the wild-type strain and the mutant. The growth rate of *V. anguillarum* was not affected by the *amiB* mutation. However, *amiB* mutant cells sedimented when grown overnight, whereas a broth culture of wild-type strain was turbid with just a few sedimented cells. As shown in Fig. 1, the colony of *amiB* mutant extended broader than that of wild-type strain on plate. TEM revealed that the *amiB* mutant formed a long chain of cells, compared with the parental strain (Fig. 2), clearly demonstrating that AmiB is involved in cell separation. Furthermore, we expected that inactivation of the *amiB* gene affected virulence factors and other physiological characteristics. However, contrary to our expectation, the wild-type strain and the *amiB* mutant did not show any significant difference in the production

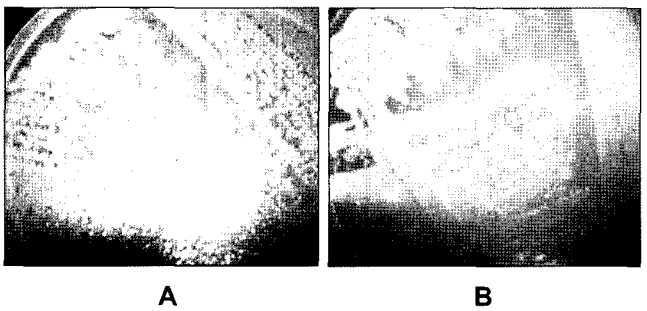

Fig. 1. Colony morphology of wild-type strain and *amiB* mutant grown on agar plate.
A. Wild-type strain; **B.** *amiB* mutant.

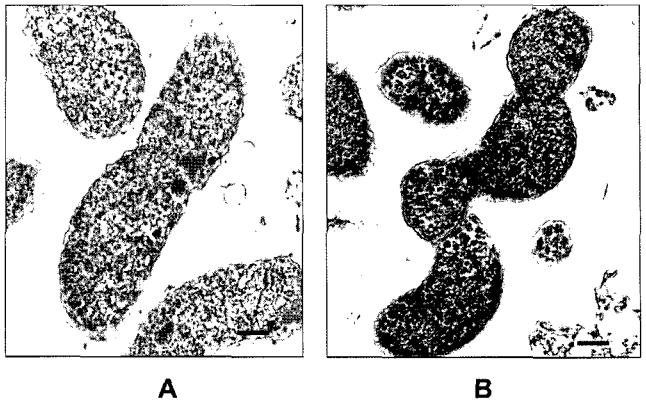

Fig. 2. Transmission electron microscopic observation of the wild-type and mutant strains.
A representative field was examined for exponential growth. **A.** Wild-type strain; **B.** *amiB* mutant. The mutant cells do not normally separate. Bars, 200 nm.

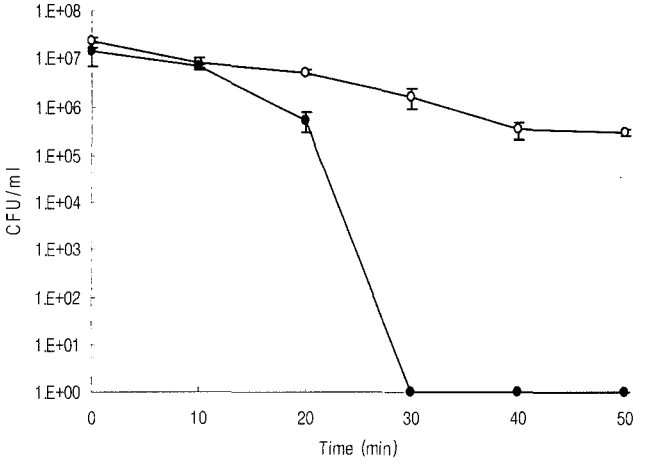

Fig. 3. Effect of exposure to H_2O_2 (100 mM) on early stationary growth phase (optical density of 1.2 at 600 nm) cells of *V. anguillarum* (-○-) and *amiB* mutant (-●-).
The values represent triplicate determinations.

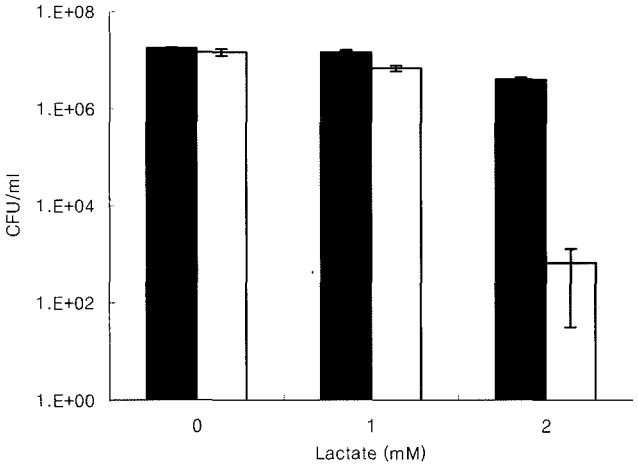

Fig. 4. Survival of *V. anguillarum* (■) and *amiB* (□) mutant after 3 h of incubation with 0, 1, and 2 mM lactate, respectively.

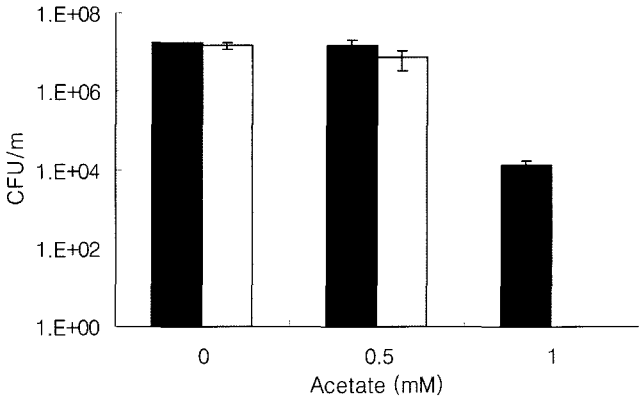

Fig. 5. Survival of *V. anguillarum* (■) and *amiB* (□) mutant after 3 h of incubation with 0, 0.5, and 1 mM acetate, respectively.

of several potential virulence factors, including hemolysin and proteases.

Oxidative Resistance

We examined the effect of H_2O_2 on the cell viability of the wild-type strain and the *amiB* mutant. The *amiB* mutant was sensitive to H_2O_2 exposure compared with the wild-type strain: As shown in Fig. 3, viable cells of the mutant strain decreased rapidly and the survival rate of the wild-type strain was 7-fold higher than the mutant strain after 20 min of incubation in 100 mM H_2O_2. After 30 min of H_2O_2 exposure, the wild-type strain was reduced by 14-fold and still persisted after 60 min of exposure, but a few cells of the mutant strain were detected.

Effect of Organic Acid

The ability for survival in the presence of organic acid was investigated. *V. anguillarum* was not significantly affected in 1 mM lactate or 0.5 mM acetate (Figs. 4 and 5, respectively). Approximately 50% of the *amiB* mutant survived after 3 h in 1 mM lactate and 0.5 mM acetate. After 3 h of incubation in 2 mM lactate, cells were rapidly killed. On the other hand, the ability of the wild-type strain to survive was much stronger than the mutant strain. Similarly, after exposure to 1 mM acetate for 3 h, 0.07% of cells of the wild-type strain survived, but the mutant strain was completely killed.

DISCUSSION

The purpose of this study was to characterize the first known autolysin involved in peptidoglycan hydrolysis of *V. anguillarum*. The *amiB* gene was identified from chromosomal DNA of *V. anguillarum* and was found to encode *N*-acetylmuramoyl-L-alanine amidase.

The sequence analysis revealed two conserved domains, Ami and repeated LysM, in AmiB of *V. anguillarum*. The role of the Ami domain in the enzyme function is to cleave the amide bond between the *N*-acetylmuramoyl moiety

A

```
231  SEIARRNKSHLLVSIHADAFHTPQPRGGSVFVLNTKRANTEIARWVENHEQQSELLGGAGEVLAKNNSDRNVSQTLLDLQFSHSQKEG  V. anguillarum
228  TEIARRSKAHLLVSVHADAFHTPQPRGGSVFVLNTRRANTEIARWVENHEQQSELLGGAGEVLSKTNNDRNVSQTLLDLQFSHSQKEG  V. cholerae
230  TEIARKNKSHLLVSIHADSFRSPQPRGGSVFVLNTRRANTEIGRWVEKHEKQSELLGGAGDVLAKNSNDRNVSQTLLDLQFSHSQKEG  V. fischeri
230  VAFARENDAHLLISVHADAFTSPQPRGGSVFVLNTRRANTEIARWVENHEKQSELLGGSGNAFVTNTKDRNVNQTLLDLQFSHSQKEG  V. parahaemolyticus
230  VAIARENDAHLLISVHADAFTSPQPRGGSVFVLNTRRANTEIARWVENHEKQSEILGGSGAAFT-NINDKNVNQTLLDLQFSHSQKEG  V. vulnificus

YKVATHLLGEMGKVAHLHKAEPVNASLAVLKSPDIPSVLVETGFISNPTEEQLLFQRSHQDKLARALATAIV----  390  V. anguillarum
YKVATNILREMGKVAHLHKTEPVNASLAVLKSPDIPSVLVETGFISNPSEEKLLIQRSHQDKLARALATAIVQYFE  391  V. cholerae
YKLAKNVLSEMGKVIRLHKKEPVNASLAVLKSPDIPSILIETGFISNPTEEKQLFQRAHQDKIARSISKAIIRYLH  393  V. fischeri
YKLATEILGEMGRVAHLHNTKPINTSLAVLRSPQIPSVLVETGFISNPTEEKLLFQRAHQDKLAQAISKAVVKYLK  393  V. parahaemolyticus
YKLATEILSEMGKVAQLHNSQPINTSLAVLRSPQIPSVLVETGFISNPTEEKLLFQRAHQDKLAKAISKAVLKYLK  392  V. vulnificus
```

B

```
repeat1  414 VQRGESLSVIANKYGTSTQALIATNKLKNANLSIGQILTI 453
repeat2  478 VKSGEYLGKIASTYKVSVDAIKRENNLRSDTLTLGQKLKI 517
repeat3  531 VEKGEFLSKIASQYQVSLNSIRQVNQLRSDQLAIGQVLII 570
```

Fig. 6. Analysis of the AmiB (*N*-acetylmuramoyl-L-alanine amidase) sequence from *V. anguillarum*.
A. Comparison of the Ami domain in *N*-acetylmuramoyl-L-alanine amidase sequence with other homologous sequences in *Vibrio* sp. **B.** Alignment of the deduced amino acid sequences of the repetitive sequences. Repeat1, Repeat2, and Repeat3, in the *N*-acetylmuramoyl-L-alanine amidase from *V. anguillarum*. Highlighted letters indicate conserved amino acids. Dots indicate conserved amino acids.

and L-amino acids in bacterial cell walls. As shown in Fig. 6A, the Ami domain of the N-terminus is very similar to those of *V. cholerae*, *V. fischeri*, *V. parahaemolyticus*, and *V. vulnificus*: The identities were 90%, 80%, 75%, and 73%, respectively. Three repeated LysM motifs of the C-terminus found in a variety of enzymes are involved in bacterial cell wall degradation. These motifs may have a general peptidoglycan binding function. Specifically, the repeated motif 2 (40 amino acid residues, starting at V-478) and motif 3 (40 amino acid residues, starting at V-531) are 55% identical, and the repeated motif 1 (40 amino acid residues, starting at V-414) shares only 42% identical with the motif 3 (Fig. 6B). This result is quite similar to another observation that the autolysin (Aae) of *Staphylococcus epidermidis* has three repetitive sequences in its N-terminal region. [9] Sequence analysis of cloned plasmid, pAMI5, showed that the *mutL* repair gene was located downstream of the *amiB* gene, and this is the same gene order as the *amiB* and *mutL* genes of *E. coli*.

V. anguillarum wild-type and *amiB* mutant have different colony morphology on plate. Mutant cell formed a broad colony on plate (Fig. 1) and sedimented in liquid medium, compared with wild-type strain cells. Electron microscopy showed that the ability for cell division of the *amiB* mutant decreased less than that of the wild-type strain (Fig. 2), suggesting that septa of the *V. anguillarum amiB* mutant are formed, but not cleaved because of the deficiency of the AmiB protein. In most Gram-negative bacteria, the division process starts before completion of septum formation [2]. This process depends on cleavage of the peptidoglycan septum that is synthesized during cell division. In *E. coli*, three amidases, named as AmiA, AmiB, and AmiC, have been reported [8]. The double *amiA* and *amiB* deletion mutant showed only a little tendency to grow in chains (5–10%), whereas the *amiA/C* and the *amiB/C* double mutants showed about 20% chains. However, the *amiA/B/C* triple deletion mutants grow in long chains of unseparated cells. The *amiB* gene of *V. anguillarum* is the first gene among *Vibrio* sp. to be reported. By analysis of known genome sequences, one or two putative *N*-acetylmuramoyl-L-alanine amidase genes were identified in *V. cholerae*, *V. fischeri*, *V. parahaemolyticus*, and *V. vulnificus*. However, it is not clear whether *N*-acetylmuramoyl-L-alanine amidases of *Vibrio* sp. consist of AmiA, AmiB, and AmiC, like *E. coli*.

When *V. anguillarum* is taken up by marine fish, the bacterial cells should encounter an acidic environment and survive during passage through the stomach. They finally attach and colonize within the intestine of the fish. In *V. cholerae*, the expression level of heat-shock proteins changes in order to maintain the homeostasis of cytoplasmic pH; about 110 proteins of *V. cholerae* have been shown to be affected by exposure to organic acids [16]. However, there is no evidence to indicate which proteins are induced or repressed, with an exception of ToxR-mediated proteins. In addition, the acid-tolerance response system is associated with outer and inner membrane porins for bacterial survival in organic acid stress [16, 19]. Heidrich *et al.* [8] reported that *E. coli* mutants with deletions of murein hydrolases, including amidases, are affected in their septum cleavage, resulting in an increase of outer membrane permeability. Compared with the wild-type strain, the mutant strain also showed susceptibility to growth inhibition and lysis by toxic molecules [8]. In this study, we compared the survival rate of the *amiB* deletion mutant constructed by the allelic exchange method in the presence of organic acids. When acetate and lactate were added to the mutant and wild strains, the survival ability of the *amiB* mutant was lower than the wild strain. These results suggested that the deletion of the *amiB* gene may induce the alteration of membrane structure, resulting in an increase of organic acid fluidity or permeability. It is also possible that mutation of the *amiB* gene leads to an increase of sensitivity to oxidative stress, by H_2O_2. Further studies are needed to determine how AmiB is involved in sensitivity to oxidative stress that can damage bacteria in the stomach of the host. Therefore, it is thought that the sensitivities of *amiB* mutant to organic acids and hydrogen peroxide may be associated with the virulence of *V. anguillarum* during host infection. Future studies involving virulence of the *amiB* mutant of *V. anguillarum* should help to clarify the role of *amiB* in the pathogenesis of this organism.

Acknowledgments

This study was supported by a grant (B-2004-10) from the Marine Bioprocess Research Center of the Marine Bio 21 Center funded by the Ministry of Maritime Affairs and Fisheries, Republic of Korea. *Vibrio anguillarum* serotype O1 was obtained from Dr. K. Holmstrøm, Biotechnolgical Institute, Department of Molecular Characterization, Denmark. Plasmid pNQ705 was kindly provided by Professor Sang Ho Choi, Seoul National University.

References

1. Berry, A. M., R. A. Lock, D. Hansman, and J. C. Paton. 1989. Contribution of autolysin to virulence of *Streptococcus pnemoniae*. *Infect. Immun.* **57:** 2324–2330.
2. Burdett, I. D. J. and R. E. G. Murray. 1974. Septum formation in *Escherichia coli*: Characterization of septal structure and the effects of antibiotics on cell division. *J. Bacteriol.* **119:** 303–324.
3. Byun, D. S., D. S. Kim, J. S. Godber, S. W. Nam, M. J. Oh, H. S. Shim, and H. R. Kim. 2004. Isolation and characterization of marine bacterium producing arylsulfatase. *J. Microbiol. Biotechnol.* **14:** 1134–1141.

4. Chatfield, C. H., H. Koo, and R. G. Quivey Jr. 2005. The putative autolysin regulator LytR in *Streptococcus mutans* plays a role in cell division and is growth-phase regulated. *Microbiology* **151:** 625–631.
5. Chen, Q. and J. H. Crosa. 1996. Antisense RNA, fur, iron, and the regulation of iron transport genes in *Vibrio anguillarum*. *J. Biol. Chem.* **271:** 18885–18891.
6. Fein, J. E. 1979. Possible involvement of bacterial autolytic enzymes in flagellar morphogenesis. *J. Bacteriol.* **137:** 933–946.
7. Fein, J. E. and H. J. Rogers. 1976. Autolytic enzyme-deficient mutants of *Bacillus subtilis* 168. *J. Bacteriol.* **127:** 1427–1442.
8. Heidrich, C., A. Ursinus, J. Berger, H. Schwarz, and J. V. Höltje. 2002. Effect of multiple deletions of murein hydrolases on viability, septum cleavage, and sensitivity to large toxic molecules in *Escherichia coli*. *J. Bacteriol.* **184:** 6093–6099.
9. Heilmann, C., G. Thumm, G. S. Chatwal, J. Hartleib, A. Uekötter, and G. Peters. 2003. Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. *Microbiology* **149:** 2769–2778.
10. Heo, M. S., J. H. Kim, S. H. Park, G. J. Woo, and H. Y. Kim. 2004. Detection of genetically modified maize by multiplex PCR method. *J. Microbiol. Biotechnol.* **14:** 1150–1156.
11. Holmstrøm, K. and L. Gram. 2003. Elucidation of the *Vibrio anguillarum* genetic response to the potential fish probiont *Pseudomonas fluorescens* AH2, using RNA-arbitrarily primed PCR. *J. Bacteriol.* **185:** 831–842.
12. Jeong, K. C., H. S. Jeong, J. H. Rhee, S. E. Lee, S. S. Chung, A. M. Starks, G. M. Escudero, P. A. Gulig, and S. H. Choi. 2000. Construction and phenotypic evaluation of a *Vibrio vulnificus vvpE* mutant for elastolytic protease. *Infect. Immun.* **68:** 5096–5106.
13. Kim, S. H., S. H. Ahn, J. H. Lee, E. M. Lee, K. J. Park, and I. S. Kong. 2003. Genetic analysis of phosphomannomutase/phosphoglucomutase from *Vibrio furnissii* and characterization of its role in virulence. *Arch. Microbiol.* **180:** 240–250.
14. Lenz, L. L., S. Mohammadi, A. Geissler, and D. A. Portnoy. 2003. SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. *Proc. Natl. Acad. Sci. USA* **100:** 12432–12437.
15. Mari, N., L. M. Baddour, D. Q. Offutt, V. Vijaranakul, M. J. Nadakavukaren, and R. K. Jayaswal. 1994. Autolysin-defective mutant of *Staphylococcus aureus*: Pathological considerations, genetic mapping, and electron microscopic studies. *Infect. Immun.* **62:** 1406–1409.
16. Merrell, D. S. and A. Camilli. 2002. Acid tolerance of gastrointestinal pathogens. *Microbiology* **5:** 51–55.
17. Milohanic, E., R. Jonquieres, P. Cossart, P. Berche, and J. L. Gaillard. 2001. The autolysin Ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. *Mol. Microbiol.* **39:** 1212–1224.
18. Navarre, W. W. and O. Schneewind. 1999. Surface proteins of Gram positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol. Mol. Biol. Rev.* **63:** 174–229.
19. Oh, E. T., Y. J. Ju, S. C. Koh, Y. H. Kim, J. S. Kim, and J. S. So. 2004. Lack of *O*-polysaccharide renders *Bradyrhizobium japonicum* more resistant to organic acid stress. *J. Microbiol. Biotechnol.* **14:** 1324–1326.
20. Ohk, S. H., S. W. Nam, J. M. Kim, Y. J. Yoo, and D. H. Bai. 2004. Purification and characterization of cell wall hydrolase from alkalophilic *Bacillus mutanolyticus* YU5215. *J. Microbiol. Biotechnol.* **14:** 1142–1149.
21. Pooley, M. M. and D. Karamata. 1984. Genetic analysis of autolysin-deficient and flagellaless mutants of *Bacillus subtilis*. *J. Bacteriol.* **160:** 1123–1129.
22. Rogers, H. J., P. F. Thurman, and I. D. J. Burdett. 1983. The bactericidal action of beta-lactam antibiotics on an autolysin-deficient strain of *Bacillus subtilis*. *J. Gen. Microbiol.* **129:** 465–478.
23. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Cold Spring Habor Laboratory Press, Cold Spring Habor, New York, U.S.A.
24. Smith, T. J., S. A. Blackman, and S. J. Foster. 2000. Autolysins of *Bacillus subtilis*: Multiple enzymes with multiple functions. *Microbiology* **146:** 249–262.
25. Toranzo, A. E., Y. Santos, and J. L. Barja. 1997. Immunization with bacterial antigens: *Vibrio* infections. *Dev. Biol. Stand.* **90:** 93–105.
26. Ward, J. B. and R. Williamson. 1984. *In* Nombela, C. (ed.). *Microbial Cell Wall Synthesis and Autolysis*, pp. 159–166. Elservier. Amsterdam.