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

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

\section*{Materials and Methods}

\subsection*{Bacterial Strains and Culture Condition}

The bacterial strains and plasmids used in this study are listed in Table 1. Brain-heart infusion (BHI; D\textit{\textregistered}i\text{\textregistered}co Laboratories) and Luria-Bertani (LB; D\textit{\textregistered}i\text{\textregistered}co Laboratories) broth and agar were used to grow \textit{V. anguillarum} and \textit{Escherichia coli} strains, respectively. Antibiotics were used at the following concentrations: 100 \(\mu\)g of ampicillin per ml for \textit{E. coli} and 15 \(\mu\)g of chloramphenicol per ml for the \textit{amiB} mutant.

\subsection*{Cloning of \textit{amiB} Gene from \textit{V. anguillarum}}

Procedures for DNA isolation, DNA digestion, ligation, agarose gel electrophoresis, and PCR were performed as described by Sambrook and Russell [23]. The cloning vector pGEM-4Z was used to generate a genomic DNA library from \textit{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].

\subsection*{Construction of \textit{amiB} Knockout Mutant}

To construct the \textit{V. anguillarum} strain deficient in the production of \textit{AmiB}, a 0.6-kb \textit{amiB} fragment (991-1587) was amplified by polymerase chain reaction (PCR) from \textit{V. anguillarum} chromosomal DNA using the primers 5'-GGCCGTCGACGGTACAAAGGTGGAACCT-3' and 5'-GGCGGACGTTCCCCACGTTAAGGTGGCTTGAA-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 pAM16 was introduced into the conjugation donor \textit{E. coli} SM10::\textit{pir}. Conjugation was carried out between the recipient \textit{V. anguillarum} and the donor \textit{E. coli} strain containing pAM16. A conjugant carrying a single-crossover mutation of \textit{amiB} was obtained by selection on thiosulfate citrate bile salts (TCBS) agar containing 15 \(\mu\)g/ml chloramphenicol, and was confirmed to have an insertion into the \textit{amiB} gene by PCR analysis.

\subsection*{Susceptibility of \textit{V. anguillarum} and \textit{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 \(\mu\)l 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.

\subsection*{Tolerance to Organic Acid}

To determine the resistance of \textit{V. anguillarum} and \textit{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 15 mM). The bacterial cells were collected and washed three times with PBS. The cell suspension was serially diluted and plated onto BHI agar plates for counting viable bacterial cells. The survival was determined 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.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Strain or plasmid} & \textbf{Reference or source} & \textbf{Relevant characteristics}\textsuperscript{a} \\
\hline
\textit{Vibrio anguillarum} & & \\
amiB mutant & & This study \\
\textit{Escherichia coli} & & \\
DH5\textalpha & & Invitrogen Co. \\
\textit{SM10::pir} & \textit{thi thr leu tonA lacY supE44 recA1 gyrA96 thi-1 relA1} & [12] \\
\hline
\textbf{Plasmids} & & \\
pGEM-4Z & Cloning vector; Ap\textsuperscript{'} & Promega Co. \\
pNQ705 & Cloning vector; R6K \textgamma ori (requires \textgamma); oriT of RP4; Cm\textsuperscript{'} & [12] \\
pAMI5 & pGEM-4Z with 5.0 kb BamHI fragment containing \textit{amiB} from \textit{V. anguillarum} & This study \\
pAM16 & pNQ705 with 0.6-kb SalI-SacI fragment internal coding sequence of \textit{amiB}; for allelic exchange & This study \\
\hline
\end{tabular}
\caption{Strains and plasmids used in this study.}
\end{table}

\textsuperscript{a}Ap\textsuperscript{'} ampicillin resistant; Cm\textsuperscript{'} chloramphenicol resistant; Km\textsuperscript{'} kanamycin 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 pAM15, 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, pNO705, 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...
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of several potential virulence factors, including hemolysin and proteases.

Oxidative Resistance
We examined the effect of H$_2$O$_2$ on the cell viability of the wild-type strain and the amib mutant. The amib mutant was sensitive to H$_2$O$_2$ 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$_2$O$_2$. After 30 min of H$_2$O$_2$ 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 and L-alanine.
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/amiC and the amiB/amiC double mutants showed about 20% chains. However, the amiB/amiC 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 H2O2. 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.

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REFERENCES


