

The Capability of Catabolic Utilization of *N*-Acetylneuraminic Acid, a Sialic Acid, Is Essential for *Vibrio vulnificus* Pathogenesis[∇]

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***N*-acetylneuraminic acid (Neu5Ac, sialic acid) could provide a good substrate for enteropathogenic bacteria in the intestine, when the bacteria invade and colonize in human gut. In order to analyze the role of Neu5Ac catabolism in *Vibrio vulnificus* pathogenesis, a mutant with disruption of the *nanA* gene encoding Neu5Ac lyase was constructed by allelic exchanges. The *nanA* mutant was not able to utilize Neu5Ac as a sole carbon source and revealed an altered colony morphotype with reduced opacity in the presence of Neu5Ac. Compared to the wild type, the *nanA* mutant exhibited a low level of cytotoxicity toward INT-407 epithelial cells in vitro and reduced virulence in a mouse model. The disruption of *nanA* also resulted in a substantial decrease in histopathological damage in jejunum and colon tissues from the mouse intestine. These results indicated that NanA plays an important role in *V. vulnificus* pathogenesis. In addition, the *nanA* mutant was significantly diminished in growth with and adherence to INT-407 epithelial cells in vitro, and was defective for intestinal colonization, reflecting the impaired ability of the mutant to grow and survive with, persist in, and adhere to the intestine in vivo. Consequently, the combined results suggest that NanA and the capability of catabolic utilization of Neu5Ac contribute to *V. vulnificus* virulence by ensuring growth, adhesion, and survival during infection.**

Microbial pathogenicity is a complex phenomenon that involves the products of many genes, called virulence factors, contributing not only to diseases but also to survival and multiplication on or within the host (20). For the development of diseases, survival and multiplication are clearly the priorities of the infecting microorganisms. It is likely that when enteropathogenic bacteria invade the human gut, many environmental changes, such as differences in types and concentrations of nutrients, would be encountered. In addition to the scarcity of specific nutrients, increased competition for the nutrient imposed by the host cells and endogenous bacterial flora would be encountered. Thus, the ability to acquire nutrients under these adverse environments is often crucial for enteropathogenic bacteria to survive and multiply in the intestine (5). For example, because the amount of free glucose would be quite small in the intestine, enteropathogenic bacteria must be able to use nutrients other than glucose to be a successful pathogen (11, 15).

Epithelial surfaces of the intestine that are exposed to bacteria are the major components of innate immunity. Therefore, bacteria have to penetrate the epithelial surfaces to invade the host. The mucus layer that overlays epithelial surfaces is the primary place where bacteria adhere and colonize. It has been observed that *Escherichia coli* mutants that have difficulty

growing on or surviving in mucus are unable to colonize the mouse intestine (25). The mucus layer is composed of a variety of factors, but its main properties are attributable to the presence of mucins, which are complex linear polymorphic glycoproteins produced mainly by goblet cells (43). Mucins are highly glycosylated large glycoproteins (with molecular weights ranging from 5×10^3 to 4×10^6 Da), and up to 85% of their dry weight is carbohydrate (42). As such, the carbohydrate component of a mucin molecule is one of the most abundant mucosal polysaccharides (42), indicating that mucin sugars are important carbon sources to support the survival and growth of infecting enteropathogens.

Sialic acid is a generic term to represent a family of related nine-carbon sugar acids that are located prominently at the terminal end of the carbohydrate side chains of mucin (for recent reviews, see references 34 and 39). The most abundant sialic acid is *N*-acetylneuraminic acid (Neu5Ac); therefore, many enteropathogenic bacteria have evolved elaborate systems for the utilization of Neu5Ac as a potential carbon and nitrogen source. However, few definitive analyses of the genes encoding the proteins involved in the degradation of Neu5Ac have been reported. The *nan* (stands for *N*-acylneuraminate) systems of *E. coli* and *Haemophilus influenzae*, consisting of many genes, including *nanA*, *nanK*, and *nanE* (or homologues), are genetic systems of which the functions are well characterized at the molecular level (38, 39, 40). In *E. coli*, the Neu5Ac lyase (NanA) initiates the catabolism of Neu5Ac by cleaving it into pyruvate and *N*-acetylmannosamine (ManNAc), which is ultimately converted into an intermediate of central metabolism (fructose 6-phosphate) via the activities of many

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TABLE 1. Plasmids and bacterial strains used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
Bacterial strains		
<i>V. vulnificus</i>		
HG071	ATCC 29307 with spontaneous Rif ^r mutation, virulent	Laboratory collection
HG072	HG071 <i>nanA::nptI</i> , Km ^r , Rif ^r	This study
<i>E. coli</i> SM10λpir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu λpir</i> ; Km ^r ; host for π-requiring plasmids; conjugal donor	22
Plasmids		
pUC4K	pUC4 with the <i>nptI</i> gene; Ap ^r , Km ^r	Pharmacia
pDM4	Suicide vector; <i>oriR6K</i> ; Cm ^r	23
pGEM-T Easy	PCR product cloning vector; Ap ^r	Promega
pJH0311	0.3-kb <i>NruI</i> fragment containing multicloning site of pUC19 cloned into pCOS5; Ap ^r , Cm ^r	7
pHG0701	pGEM-T Easy with the <i>nanA</i> gene; Ap ^r	This study
pHG0703	pDM4; <i>nanA::nptI</i> ; Cm ^r , Km ^r	This study
pHG0704	pJH0311 with the <i>nanA</i> gene; Ap ^r , Cm ^r	This study

^a Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Rif^r, rifampin resistant.

proteins, including NanK and NanE (39). Nevertheless, until now, very little has been known about the role of Neu5Ac degradation genes in the pathogenesis of bacteria (40).

The pathogenic marine bacterium *Vibrio vulnificus* is the causative agent of food-borne diseases, such as gastroenteritis and life-threatening septicemia in immunocompromised individuals (for recent reviews, see references 8, 18, and 37). A search of the GenBank database (<http://www.ncbi.nlm.nih.gov>) for homology to the amino acid sequence of *E. coli* NanA singled out the putative *V. vulnificus* NanA protein, suggesting that *V. vulnificus* is able to utilize Neu5Ac as a nutrient during infection. However, no studies have yet been reported on the effects of the capability of catabolic utilization of Neu5Ac on *V. vulnificus* virulence. Accordingly, in the present study, a *V. vulnificus* null mutant, in which the *nanA* gene was disrupted and thus is not able to utilize Neu5Ac as a nutrient, was constructed by allelic exchange. In order to demonstrate the possible roles of the catabolic utilization of Neu5Ac in *V. vulnificus* pathogenesis, the adhesion, survival, and multiplication as well as virulence of the *nanA* mutant were compared with those of the wild type by using tissue cultures and mice. It appeared from the results that NanA is essential for *V. vulnificus* virulence by ensuring adhesion, survival, and multiplication during infection.

MATERIALS AND METHODS

Strains, plasmids, and culture media. The strains and plasmids used in this study are listed in Table 1. *V. vulnificus* ATCC 29307 with spontaneous rifampin (rifampicin) resistance was named HG071 and used as a wild type. Unless noted otherwise, the *V. vulnificus* strains were grown in Luria-Bertani medium supplemented with 2.0% (wt/vol) NaCl (LBS) at 30°C. All the medium components were purchased from Difco (Detroit, MI), and the chemicals were purchased from Sigma (St. Louis, MO).

Cloning of *V. vulnificus nanA* and generation of the *nanA* mutant. A whole *nanA* open reading frame was amplified from the genomic DNA of *V. vulnificus* HG071 by PCR using a pair of oligonucleotide primers, NANA001 (5'-TCTAG AATGATGAACAAATTAAGG-3') and NANA002 (5'-CTCGAGCTACA GATTAAGAAAATCC-3'). The primers were designed using the genomic sequence of *V. vulnificus* (GenBank accession number VV2_0730). The amplified 900-bp *nanA* was ligated into pGEM-T Easy (Promega, Madison, WI) to result in pHG0701 (Table 1).

To inactivate *nanA* in pHG0701 in vitro, a 1.2-kb *nptI* DNA conferring resistance to kanamycin (26) was inserted into a unique *PvuI* site present 314 bp away

from the translational initiation codon of *nanA*, and the resulting 2.1-kb *nanA::nptI* mutant was ligated with *XbaI*-*XhoI*-digested pDM4 (23) to form pHG0703 (Table 1). *E. coli* SM10 *λpir tra* (containing pHG0703) (22) was used as a conjugal donor to *V. vulnificus* HG071 to generate the *nanA* mutant by homologous recombination. The conjugation and isolation of the transconjugants were conducted using methods previously described (13), and a resulting *nanA::nptI* mutant chosen for further analysis was named HG072 (Table 1).

Neu5Ac lyase activities. Neu5Ac lyase activities were measured using the method described by Bulai et al. (4) with modifications. Briefly, *V. vulnificus* cells grown to an A_{600} of 0.5 with M9 broth (32) supplemented with 10 mM D-xylose, 10 mM L-proline, and 5 mM Neu5Ac were harvested, disrupted by sonication (48), and spun down by centrifugation to obtain clear cytosol. For the measurement of Neu5Ac lyase activity, the reaction was initiated by the addition of 500 μ l of the cytosol (about 16 μ g of total proteins) as an enzyme source to 25 μ l of 32 mM Neu5Ac in aqueous solution and 75 μ l of 1 M potassium phosphate buffer, pH 7.4. After incubation at 37°C for 2 h, the reaction was stopped by heating at 96°C for 3 min, and the liberated ManNAc was determined by a colorimetric method using *p*-dimethylamino-benzaldehyde as described elsewhere (4, 31). One unit of Neu5Ac lyase activity is defined as the amount that releases 1 μ mol of ManNAc per min under the reaction conditions used. Protein concentrations were determined by the method used by Bradford (3), with bovine serum albumin as the standard.

Colony opacity. The wild type and the *nanA* mutant were grown overnight, and subsequently, equal amounts of the strains were spotted onto the modified M9 medium used above and solidified with 1.5% agar (Bacto agar; Difco). The plates were incubated at 30°C for 24 h, and colony morphotypes were photographed by using a UMAX digital imaging system (UTA-1100; UMAX Technologies, Inc., Fremont, CA).

Utilization of Neu5Ac as a sole carbon source. The ability of *V. vulnificus* strains to use Neu5Ac as a sole carbon source was assayed by measuring growth in the M9 broth supplemented with either 5 mM Neu5Ac or 5 mM glucose. Fifty-milliliter cultures of the modified M9 broth in 250-ml Erlenmeyer flasks were inoculated with an initial cell density (A_{600}) of approximately 0.005 and incubated at 30°C with shaking. The inocula were from late-exponential-phase cultures in LBS.

Cytotoxicity assay. Cytotoxicity was evaluated by the quantification of cytoplasmic lactate dehydrogenase (LDH) activity released by damage of plasma membranes (44). Preparation of INT-407 (ATCC CCL-6) human intestinal epithelial cells and infection with *V. vulnificus* strains were performed as described previously (28). Cytotoxicity was then determined by measuring the activity of LDH in the supernatant using a cytotoxicity detection kit (Roche, Mannheim, Germany) and expressed using the total LDH activity of the cells completely lysed by 1% Triton X-100 as 100%.

LD₅₀ determination. A group of six 7-week-old female mice (ICR specific pathogen-free; Seoul National University) was injected intraperitoneally with 100- μ l serial dilutions of the *V. vulnificus* suspensions as described elsewhere (28). Mice were overloaded with iron immediately before injection of bacterial cells. The infected mice were observed for 24 h, and the 50% lethal doses (LD₅₀s) were calculated using a method described by Reed and Muench (30). All

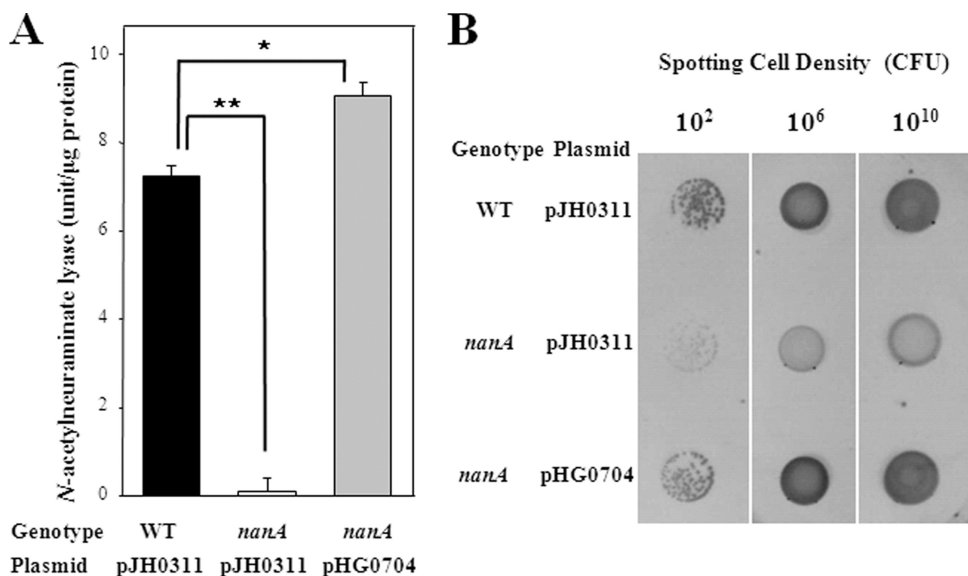


FIG. 1. Effect of the *nanA* mutation on the Neu5Ac lyase activity and colony morphotypes. Cultures of the wild type (WT) and the *nanA* mutant HG072 (*nanA*) were grown in modified M9 broth (A) or on a plate (B) containing 10 mM D-xylose, 10 mM L-proline, and 5 mM Neu5Ac. (A) Relative activities of Neu5Ac lyase were determined from samples removed at an A_{600} of 0.5 as described in the text. Error bars represent the SEM. *, $P \leq 0.01$; **, $P \leq 0.005$. (B) Colony morphotypes of the strains after 24 h of growth. Complementation of the mutant with a functional *nanA* (pHG0704) is also presented as indicated.

manipulations of mice were approved by the Animal Care and Use Committee at Seoul National University.

Histopathological examination. The female ICR mice were given drinking water containing rifampin (50 μg/ml) for 24 h to eliminate resident bacteria (21). After an 18-h starvation period without food and water, 100 μl of the bacterial inoculum, representing approximately 10^6 CFU of either the wild type or HG072, was given intragastrically to the mice. At 18 h following infection, the mice were killed, and segments taken from the jejunum and colons were sectioned transversely in their entirety and fixed overnight in 10% neutral buffered formalin. The fixed tissues were processed in paraffin, cut into five microsections, stained with hematoxylin-eosin, and examined by light microscopy.

Bacterial growth rates during infection. The INT-407 cells were infected using the wild type or HG072 at a multiplicity of infection (MOI) of 10, and growth rates of the bacterial strains during the infection were monitored. For this purpose, the INT-407 cells were broken by treatment with 0.1% Triton X-100 for 5 min at regular intervals, and bacterial cells were recovered and enumerated as numbers of CFU on LBS agar plates.

Adhesion assay. The INT-407 cell monolayer was seeded on glass coverslips placed at the bottom of the tissue culture plate and infected with the *V. vulnificus* strains at an MOI of 10 for 1 h. The monolayer was then washed two times with prewarmed phosphate-buffered saline (PBS) to remove nonadherent bacteria (17, 28). Following the last wash, the INT-407 cells were fixed in methanol, stained with 0.4% Giemsa, and examined under a light microscope (28). To quantify the adherent bacteria, the INT-407 cells were also broken after washing, and the adherent bacteria were recovered and enumerated as described above. The mean number of adherent bacteria per cell was used to represent the adhesion index of the strains.

Competition assay. Colonization activities of the wild type and *nanA* mutant HG072 were determined with the mouse model by competition assays as described earlier (24, 41). Briefly, four female ICR mice (7 weeks old) were infected as described above for the histopathological examination, except that 100 μl of the bacterial inoculum, prepared by mixing the wild type and the *nanA* mutant at a 1:1 ratio, representing approximately 10^6 CFU of each strain, was given intragastrically to the mice. Their intestines were collected after a 1- to 48-h infection, washed, and homogenized. Equal amounts of the homogenates were spread on LBS agar containing either rifampin (100 μg/ml) alone to enumerate the sum of wild-type and *nanA* mutant cells or rifampin and kanamycin (100 μg/ml) to specifically count the *nanA* mutant cells. The ratio of the number of CFU recovered from the intestines to the number of CFU inoculated is defined as the colonization index.

Data analysis. Averages and standard errors of the mean (SEM) were calculated from at least three independent experiments. The statistical significance of the difference among the *V. vulnificus* strains was evaluated using analysis of variance, and significance was accepted at P values of <0.005 .

RESULTS

Identification of the *nanA* gene and construction of the *nanA* mutant of *V. vulnificus*. The amino acid sequence deduced from the putative *nanA* nucleotide sequence revealed a protein, a putative NanA composed of 299 amino acids with a theoretical molecular mass of 32,199 Da and a pI of 5.51. The amino acid sequence of the *V. vulnificus* NanA was 26% identical to that of the *E. coli* and *H. influenzae* NanA, and this identity appeared evenly throughout the proteins (data not shown; <http://www.ncbi.nlm.nih.gov>). The predicted hydrophobicity profile (<http://www.expasy.ch>) was similar to that of the NanA of *E. coli* and *H. influenzae* and was consistent with the fact that the NanA protein is a cytosolic soluble protein (data not shown). To examine the role of NanA, the *V. vulnificus nanA* isogenic mutant HG072 (Table 1) was constructed by allelic exchanges, and the insertional disruption of the *nanA* gene in HG072 was confirmed by PCR as described previously (data not shown; 13).

Effect of the *nanA* mutation on Neu5Ac lyase activity and colony morphotypes. For the wild type, Neu5Ac lyase, initiating the catabolic degradation of Neu5Ac, was produced and reached a maximum at 7.2 units (Fig. 1A). The disruption of *nanA* in the mutant HG072 resulted in the complete loss of Neu5Ac lyase activity. These results demonstrated that the *nanA* gene encoded the *V. vulnificus* Neu5Ac lyase. The observation that the level of Neu5Ac lyase activity in the *nanA*

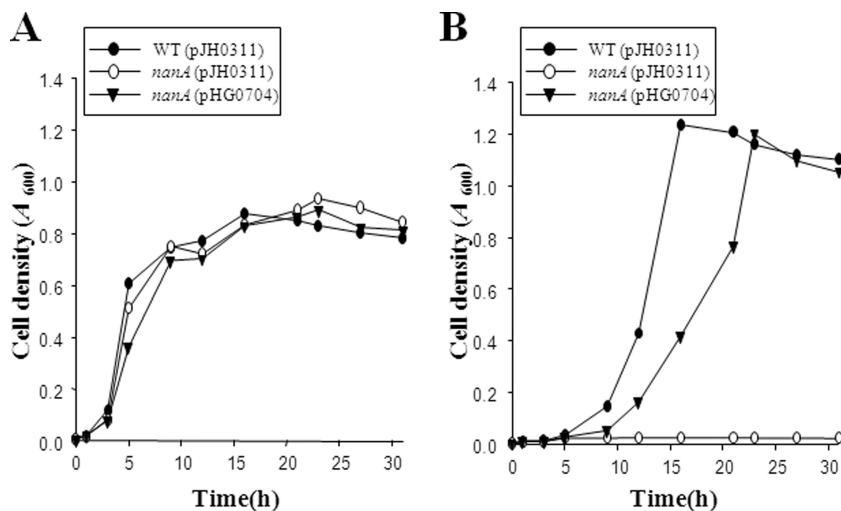


FIG. 2. Growth kinetics of the *V. vulnificus* strains. Cultures of wild-type HG071, the *nanA* mutant HG072, or the complemented strain were grown in M9 medium supplemented with 5 mM glucose (A) or 5 mM Neu5Ac (B) as a sole carbon source. The data are the means from three independent experiments. WT, wild type; *nanA* (pJH0311), *nanA* mutant; *nanA* (pHG0704), complemented strain. Details are described in Materials and Methods.

mutant was almost undetectable revealed the existence of only one Neu5Ac lyase being produced by *V. vulnificus*.

We examined whether the reintroduction of pHG0704 carrying a recombinant *nanA* could complement the decrease of Neu5Ac lyase activity of HG072. For this purpose, pHG0704 was constructed by subcloning the *nanA* amplified by a PCR using the primers NANA003 (5'-GAGCTCTGGCTACGGCCAGCCTTGG-3') and NANA004 (5'-CCCGGGATCAAAAGCCACTGGTTTTTAATA-3') into the broad host-range vector pJH0311 (7; Table 1). The Neu5Ac lyase activity of HG072 (pHG0704) was restored to a level comparable to the wild-type level of HG071 (Fig. 1A). Therefore, the decreased Neu5Ac lyase activity of HG072 was confirmed to result from the inactivation of functional *nanA* rather than any polar effects on genes downstream of *nanA*.

Morphotypes of *V. vulnificus* colonies are divided into opaque and translucent due to differences in the light transmission of the colonies. The wild-type HG071 was observed as an opaque colony type, and disruption of the *nanA* gene converted the wild type to a translucent colony type in the presence of Neu5Ac (Fig. 1B). The colony opacity of the complemented strain HG072 (pHG0704) was restored to the wild-type level (Fig. 1B), indicating that the *nanA* gene seems to contribute to the formation of the *V. vulnificus* opaque morphotype in the presence of Neu5Ac.

Effect of the *nanA* mutation on utilization of Neu5Ac as a sole carbon source. Growth was determined for the wild type, HG072, and HG072 (pHG0704) in M9 broth containing either 5 mM glucose or 5 mM Neu5Ac as a sole carbon source (Fig. 2). No significant differences were observed between the wild type, HG072, and HG072 (pHG0704) in their growth with M9 medium containing glucose (Fig. 2A). When Neu5Ac was used as a sole carbon source, 5 mM Neu5Ac supported the growth of the wild type and the complemented strain, and the stationary-phase yield of cells was even higher than that obtainable when glucose was used (Fig. 2B). In contrast to this result, HG072 that is deficient of functional NanA was not able to

grow at all, indicating that the conversion of Neu5Ac into ManNAc is essential for the growth of *V. vulnificus* with Neu5Ac as a sole carbon source.

NanA is important for cytotoxicity toward epithelial cells in vitro. Monolayers of INT-407 cells infected with the wild type, HG072, and HG072 (pHG0704) strains at different MOIs were incubated for 2 h, after which the LDH-releasing activities were determined (Fig. 3A). The *nanA* mutant HG072 exhibited significantly less LDH-releasing activity when the MOIs were 10, 20, and 50. The level of LDH activity released from the INT-407 cells infected with HG072 was almost sixfold less than that from the cells infected with the wild type at an MOI of 10. Also, the LDH activities displayed from INT-407 cells infected at an MOI of 10 were compared at different incubation times as shown in Fig. 3B. When incubated for 2 h and 3 h, the cells infected with HG072 exhibited lower LDH activities than those of the cells infected with the wild type. The lower LDH activities were restored to the level obtained from the cells infected with the wild type when the cells were incubated with HG072 (pHG0704) (Fig. 3). These results suggest that NanA is important in regard to the ability of *V. vulnificus* to infect and injure host cells.

NanA is important for virulence in mice. Predisposed individuals, i.e., those with underlying conditions related to their being immunocompromised, such as liver damage and excess levels of iron, are susceptible to infection with the pathogen *V. vulnificus* (18, 37). Therefore, mice were overloaded with iron to mimic *V. vulnificus* pathogenesis. The LD₅₀s in the iron-overloaded mice after intraperitoneal infection with *V. vulnificus* strains were measured. For six iron-treated mice in each inoculation group and with inoculations ranging from 10⁰ to 10⁸ CFU in 10-fold increments, the intraperitoneal LD₅₀ for the *nanA* mutant HG072 was 7.01 × 10⁴ CFU, compared with an LD₅₀ of 3.16 × 10¹ CFU for the wild type. Therefore, for the mouse model of intraperitoneal infection, in which the *nanA* mutant exhibited a >3-log increase in the LD₅₀ over that of the isogenic parental strain, the *nanA* mutant appeared to

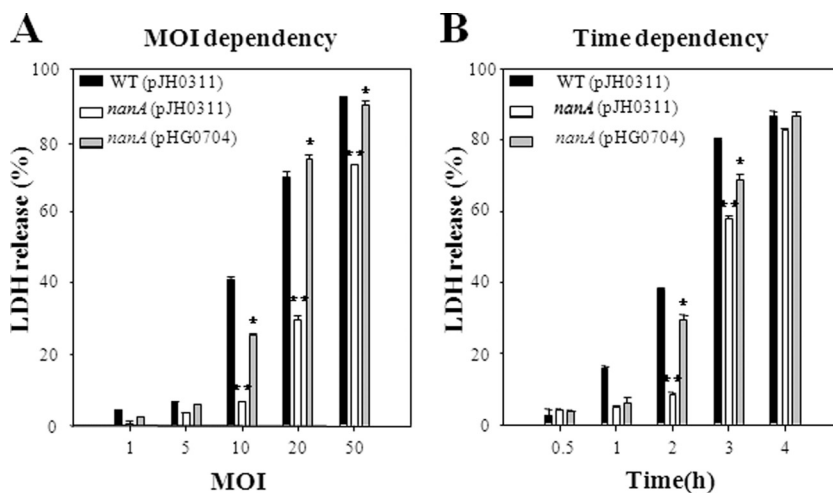


FIG. 3. Effect of the *nanA* mutation on virulence of *V. vulnificus* to INT-407 cells. INT-407 cells were infected with wild-type HG071, the *nanA* mutant HG072, or the complemented strain at various MOIs for 2 h (A) or at an MOI of 10 for various incubation times (B). Error bars represent the SEM. *, $P < 0.05$; **, $P < 0.005$ relative to groups infected with the *V. vulnificus* wild type at each MOI or incubation time. WT, wild type; *nanA* (pJH0311), *nanA* mutant; *nanA* (pHG0704), complemented strain.

be less virulent than its parental wild type. From this difference in LD₅₀s, it is apparent that *V. vulnificus* NanA is important for *V. vulnificus* virulence in mice.

Histopathology of mice infected with *V. vulnificus*. In order to understand the pathological changes occurring in the mouse intestine during infection with the wild type or HG072, histological examinations of intestinal sections of the jejunum (Fig. 4A, C, and E) and colons (Fig. 4B, D, and F) were performed. There were no histopathological changes in the jejunum (Fig. 4A) and colons (Fig. 4B) of mice that received PBS alone as a control. In comparison to the PBS-treated mice, the wild-type-treated mice revealed remarkably shorter villus lengths in the jejunum (Fig. 4C). In contrast, the HG072-treated mice had villi of lengths that were intermediate, between those of the control and wild-type-treated mice (Fig. 4E). Both wild-type and HG072-treated mice showed hypertrophy of the mucosal glands in their colons (Fig. 4D and 4F). However, mucosal glands in the colons of wild-type-treated mice (Fig. 4D) showed much greater hypertrophy than those in the colons of HG072-treated mice (Fig. 4F). Thus, these results indicated that NanA is important for the virulence of the bacteria in the mouse intestine.

Growth rates of the *V. vulnificus* strains during infection and toxic effects of Neu5Ac. When taken together, the results presented above clearly demonstrated that the NanA protein required for *V. vulnificus* to utilize Neu5Ac as a carbon source is important for *V. vulnificus* pathogenesis. However, it is unlikely that NanA could directly injure the host (or host cells), as proposed for hemolysin, elastolytic protease, and RtxA (10, 16, 36). To examine whether reduced cytotoxicity of the *nanA* mutant (Fig. 3) resulted from defects in its growth, we compared the growth rate of the *nanA* mutant with that of the wild type. The growth rate of the *nanA* mutant in minimal essential medium (MEM) with 1% fetal bovine serum in the absence of INT-407 cells was not significantly different from that of the wild type (Fig. 5A). During the infection, however, the growth rate of the *nanA* mutant in the INT-407 tissue cultures was

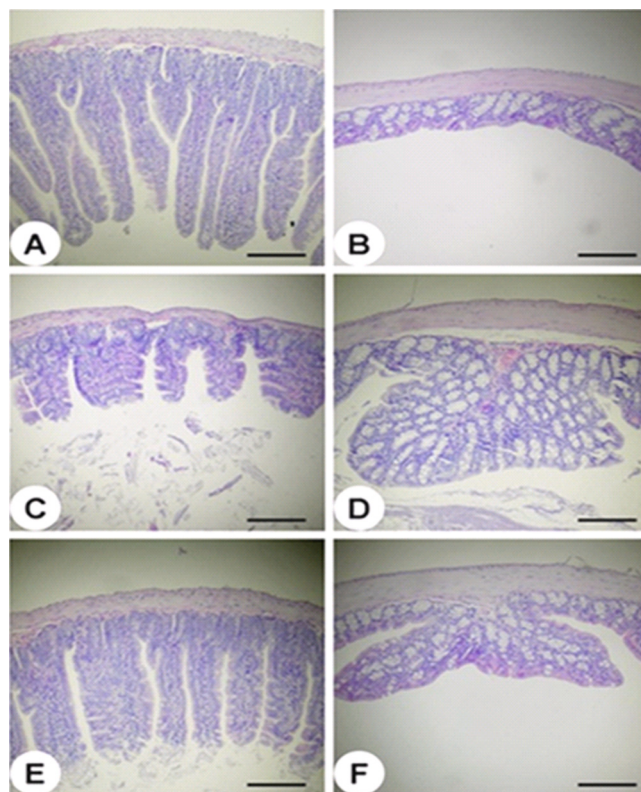


FIG. 4. Histopathology of mouse intestine tissues after infection with the *V. vulnificus* strains. The jejunum (A, C, and E) and colon (B, D, and F) tissues of mice infected intragastrically with PBS (A and B), wild-type *V. vulnificus* (C and D), or the *nanA* mutant strain of *V. vulnificus* (E and F) were histopathologically examined. Hematoxylin and eosin stain. Scale bars = 200 μm .

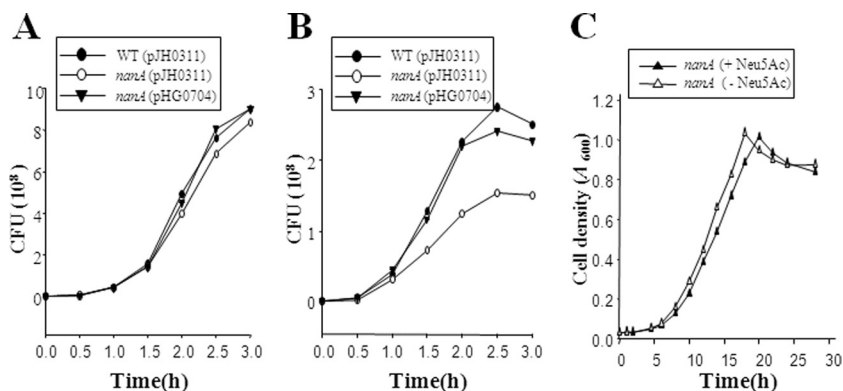


FIG. 5. Growth rates of the *V. vulnificus* strains during infection and toxic effects of Neu5Ac. (A) Growth of the strains in MEM supplemented with 1% fetal bovine serum, in the absence of INT-407 cells, was determined by enumerating CFU on LBS agar plates at the indicated time intervals. (B) For growth of the strains during infection of INT-407 cells, the strains were used to infect the INT-407 cells at an MOI of 10 and then bacterial cells during infection were enumerated as described above. (C) Cultures were grown in modified M9 broth containing 10 mM D-xylose and 10 mM L-proline and supplemented either with or without 5 mM Neu5Ac as indicated. Growth was monitored by measuring the A_{600} of the cultures. WT, wild type; *nanA* (pJH0311), *nanA* mutant; *nanA* (pHG0704), complemented strain. The data are means from three independent experiments.

significantly lower than that of the wild type (Fig. 5B), indicating that the capability of using Neu5Ac from the INT-407 cells as a carbon source makes the growth rates different. Similar results revealing the difference between growth rates of the wild type and the *nanA* mutant during infection were also obtained when the bacteria were enumerated in the supernatant without using Triton (data not shown). Therefore, it is unlikely that growth of the *nanA* mutant is more strongly inhibited by (the factors released from) the host cells than is the growth of the wild type.

In *E. coli*, growth of a *nanA* mutant is inhibited by the toxic accumulation of intracellular Neu5Ac (39). Therefore, the toxic effect of Neu5Ac on growth of the *V. vulnificus nanA* mutant was measured *in vitro*, and the result demonstrated that growth of the *V. vulnificus nanA* mutant was not inhibited substantially by the presence of Neu5Ac in the growth medium (Fig. 5C). These results combined suggest that the decreased virulence of the *nanA* mutant likely resulted from its growth defect when it infected the INT-407 cells and that the growth defect might be caused by its inability to utilize Neu5Ac rather than by a toxic accumulation of Neu5Ac.

NanA is required for adhesion to epithelial cells *in vitro*. It has been reported that the change of colony morphotypes influences the surface hydrophobicity and surface charge of the bacterial cell, and altering the physicochemical characteristics of the cell surface has been postulated to modify the relative adhesive properties of bacteria (2, 19, 46). The adhesion abilities of the *V. vulnificus* strains were compared using INT-407 monolayers. The wild type and the complemented strain HG072 (pHG0704) exhibited formation of small clusters of aggregated bacteria on the surface of INT-407 cells (Fig. 6A). After 1 h of infection, the wild type and the complemented strain adhered to INT-407 cells and reached adhesion indexes of 30 and 26, respectively (Fig. 6B). In contrast, a much smaller area of the intestinal cell surface was found covered with the *nanA* mutant HG072, and no clusters of aggregated bacteria were observed (Fig. 6A and 6B). When infected for 1 h, the number of *nanA* mutants per cell on INT-407 monolayers was

about twofold lower than that of the wild type (Fig. 6B), indicating that the *nanA* mutant was significantly impaired in its ability to attach to epithelial cells. These results suggest that NanA might contribute to *V. vulnificus* virulence by facilitating adhesion on host epithelial cells.

NanA is required for intestinal colonization *in vivo*. Because NanA is obviously crucial for the growth and adhesion of *V. vulnificus* during infection of INT-407 cells *in vitro*, it is reasonable to hypothesize that NanA also might play a similar role *in vivo* during infection of mice. To address this hypothesis, mice were coinoculated intragastrically with the *nanA* mutant and the wild type, and the strains colonized on the intestine were recovered and enumerated (Fig. 7). Both strains appeared to colonize the mouse intestine as early as 1 h after inoculation, and the colonization index increased as the period of colonization was prolonged and reached a maximum level at 18 h. The colonization index of the *nanA* mutant ranged from 10^{-5} to 10^{-3} and was consistently and significantly (about 100-fold) lower than that of the wild-type strain, demonstrating that the *nanA* mutant is severely defective in colonizing the intestines of mice.

DISCUSSION

Sialic acids are a family of over 40 naturally occurring N- or O-substituted neuraminic acids, and they mediate a wide range of biological processes, including cell-cell and cell-small molecule interactions (39). Sialic acid is also the name for the most abundant and the best-studied sialic acid, Neu5Ac. Sialic acids are found widely distributed in animal tissues, especially in mucus glycoproteins, such as mucins. The mucins are the main component of the mucus layer, which is faced consistently and persistently with invading bacteria. Therefore, it is perhaps not surprising that sialic acids play an important role in mediating host-pathogen interactions. It appears that many pathogenic bacteria have evolved multiple routes to capture sialic acid from environments and to incorporate the sialic acid into cell surface macromolecules that modulate the pathogen's interac-

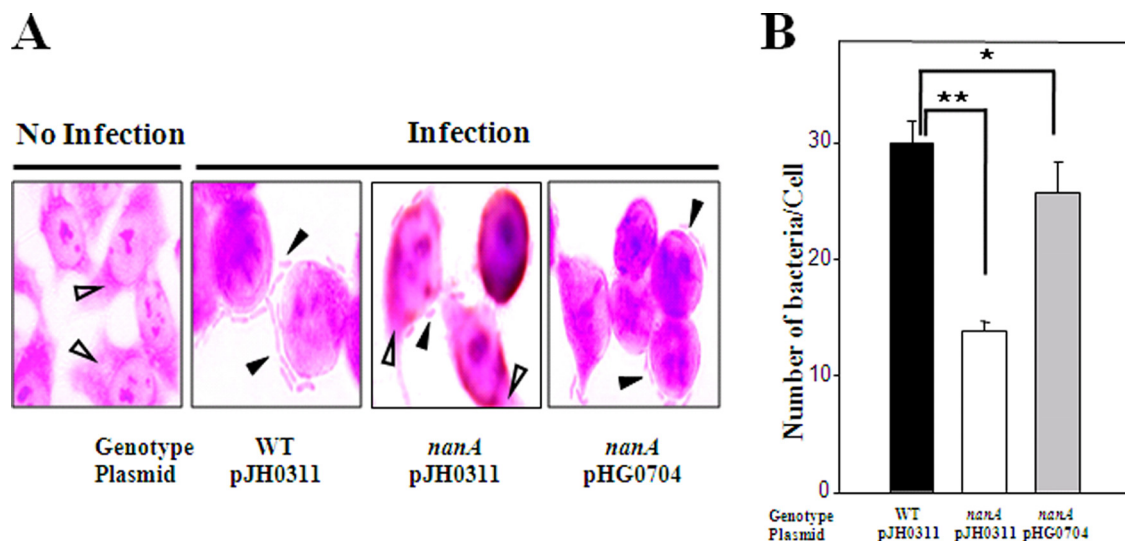


FIG. 6. Adhesion of the *V. vulnificus* strains. (A) INT-407 cells were cultured on glass coverslips and infected at an MOI of 10. After incubation with the bacteria for 1 h, the INT-407 monolayers were rinsed to remove any nonadhering bacteria. Light micrographs (original magnification, $\times 1200$) show the adhesion of the wild type, the *nanA* mutant, and the complemented strain to the INT-407 cells. The adhered *V. vulnificus* cells (closed arrowheads) and the cytoplasm of the INT-407 cells (open arrowheads) are indicated. (B) The adherent bacteria were quantified and expressed as the number of bacteria per cell in the tissue culture. *, $P < 0.05$; **, $P < 0.005$. WT, wild type; *nanA* (pJH0311), *nanA* mutant; *nanA* (pHG0704), complemented strain. Error bars represent the SEM.

tion with the host (34, 39, 40). The incorporation of sialic acid (sialylation) into bacterial cell surface molecules (such as sialylated lipopolysaccharide or polysialic acid capsule) is presumed to allow pathogens to disguise themselves as host cells and thus evade or counteract the host's immune responses (9, 34, 40). Although there is a great diversity in compositions and structure of the *V. vulnificus* capsular polysaccharide (CPS)

(29, 33), researchers have been not successful in finding Neu5Ac residues in the CPS. But the genes involved in biosynthesis of Neu5Ac and the polysialic acid capsule, such as *neuB* and *neuA*, are found for *V. vulnificus* CMCP6 and YJ016 in the GenBank genome sequence database, indicating that *V. vulnificus* can synthesize Neu5Ac and the polysialic acid capsule. However, the question of whether Neu5Ac accumulates

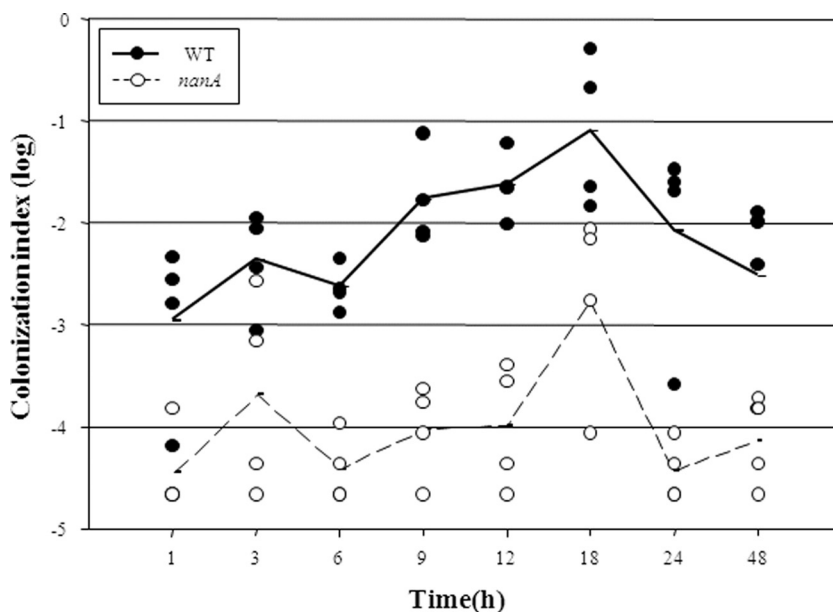


FIG. 7. Colonization dynamics of the *V. vulnificus* strains. A competition assay was performed by inoculation of a mouse with the bacterial suspension prepared by mixing equal numbers of the wild type and the *nanA* mutant and then by enumeration of the bacterial cells colonized on the mouse intestine at the indicated time intervals. Each filled (wild type) and open (*nanA* mutant) circle represents a colonization index value (number of CFU recovered/number of CFU inoculated) calculated from the result of the individual competition assay; mean values are displayed as a line on the graph.

on its surface to evade the host immune system has not yet been addressed.

Many pathogenic bacteria also utilize sialic acid as carbon, nitrogen, and energy sources (35, 39, 40). In the present study, the growth rate of the *V. vulnificus nanA* mutant in MEM was not significantly different from that of the wild type. However, the growth rate of the mutant in the INT-407 tissue culture was significantly lower than that of the wild type. This result suggests that *V. vulnificus*, as an enteropathogenic bacterium, is able to metabolize host sialic acid in those growth environments. However, until now, there has been no definitive analysis of the biochemical pathway of sialic acid metabolism in *V. vulnificus*, and the genes encoding the components of the pathways have not yet been characterized at a molecular level. Extensive searches for amino acid sequences similar to those of the *E. coli nan* system predicted the putative *nan* system from *V. vulnificus* entries in the genome sequence database (data not shown; GenBank accession numbers AE016795 and AE016796). Although the *nanA* and *nanEK* genes encoding ManNAc-6-P epimerase and ManNAc kinase are organized in the same orientation in *E. coli* (39), the presumed *V. vulnificus nanEK* is transcribed divergently from *nanA*. The differences in *nanAEK* genetic organization, along with the low level of homology in NanA amino acid sequences (26% identical), indicate that the sialic acid catabolism of *V. vulnificus* would be evolved through its own host-microbe interactions that might be different from those for *E. coli*. This lack of overall homology in genetic organizations may be a common feature inherited in the various bacterial *nan* systems as proposed by Vimr and colleagues (39).

In the present study, the *nanA* mutant that is not able to catabolically utilize Neu5Ac revealed reduced virulence and impaired growth and adhesion in tissue cultures (Fig. 3 to 6). Furthermore, the *nanA* mutant revealed a reduced colonization index (Fig. 7), reflecting the impaired ability of the mutant to not only grow, survive, and persist in but also adhere to the intestine (5). Growth of the *nanA* mutant was not inhibited by the presence of Neu5Ac in the growth medium (Fig. 5C), demonstrating that the growth defect of the *nanA* mutant may result from its inability to utilize Neu5Ac rather than a toxic accumulation of Neu5Ac. Expression of the *V. vulnificus nan* system, including the *nanT* gene encoding a Neu5Ac transporter, is tightly repressed by NanR, as is that of the *H. influenzae nan* system (S. H. Choi, unpublished data; 14). Growth of the *V. vulnificus nanR* mutant was inhibited by the presence of Neu5Ac (data not shown), indicating that the derepression of *nanT* accumulates Neu5Ac to the level of toxicity. Although other explanations are possible, it is reasonable to hypothesize that the intracellular level of Neu5Ac in the wild type and the *nanA* mutant is precisely controlled by NanR and that the toxicity of the Neu5Ac is not apparent.

We examined colony opacity of *V. vulnificus* grown with LBS and M9 medium (plus glucose) in the presence or absence of Neu5Ac. The colony morphotypes of the strains were all opaque and not significantly different from each other regardless of the presence of Neu5Ac (data not shown). Although it has been observed that colony opacity of the bacteria and the production of CPS are directly related, very little has been known about the biosynthetic pathway for the *V. vulnificus* CPS (12, 27, 28, 45, 49). However, *Pseudomonas aeruginosa* and *E.*

coli, for which CPS synthesis is well characterized biochemically and genetically, use versatile pathways for the synthesis of CPS (1, 39). Therefore, it is perhaps not surprising that *V. vulnificus* also uses versatile pathways (and versatile substrates, such as glucose) and does not depend entirely on Neu5Ac for the synthesis of CPS.

If a biosynthetic pathway similar to that for *E. coli* and *P. aeruginosa* (1, 39) operates in *V. vulnificus*, ManNAc, the product of Neu5Ac cleavage by NanA, would be converted to ManNAc-6-P and, ultimately, to UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc), which is the main activated precursor of surface-associated carbohydrate (such as CPS and lipopolysaccharide) synthesis (1, 6). One possible explanation is that loss of the ability to degrade Neu5Ac to ManNAc in the *nanA* mutant can impair the optimal synthesis of CPS and change its morphology to reduced opacity in the presence of Neu5Ac, as shown in Fig. 1B. It is generally believed that less CPS are produced in bacteria with reduced opacity, and the adhesion abilities of bacteria are affected by the amount of CPS (12, 47). Therefore, it is likely that reduced opacity may be a reason, if not the sole reason, for the impaired adherence exhibited by the *nanA* mutant (Fig. 6). However, the exact role of NanA in CPS biosynthesis and alteration of adhesion activity of *V. vulnificus* still remains to be determined.

In summary, it is apparent that *V. vulnificus* is able to utilize Neu5Ac as a nutrient during infection. The *nanA* mutant that was not able to catabolically utilize Neu5Ac revealed a less-opaque colony morphology and exhibited a reduced virulence in tissue cultures and in mice, indicating that NanA plays an important role in *V. vulnificus* pathogenesis. In addition, growth with and adherence to epithelial cells and intestinal colonization of the *nanA* mutant were significantly impaired, indicating that NanA and the capability of catabolic utilization of Neu5Ac could contribute to *V. vulnificus* pathogenesis by ensuring survival and multiplication during infection rather than directly aggravating damage or injury of the host.

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