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Sialic Acid Catabolism Confers a Competitive Advantage to Pathogenic *Vibrio cholerae* in the Mouse Intestine▽

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Sialic acids comprise a family of nine-carbon ketosugars that are ubiquitous on mammalian mucous membranes. However, sialic acids have a limited distribution among *Bacteria* and are confined mainly to pathogenic and commensal species. *Vibrio* pathogenicity island 2 (VPI-2), a 57-kb region found exclusively among pathogenic strains of *Vibrio cholerae*, contains a cluster of genes (*nan-nag*) putatively involved in the scavenging (*nanH*), transport (*dctPQM*), and catabolism (*nanA*, *nanE*, *nanK*, and *nagA*) of sialic acid. The capacity to utilize sialic acid as a carbon and energy source might confer an advantage to *V. cholerae* in the mucus-rich environment of the gut, where sialic acid availability is extensive. In this study, we show that *V. cholerae* can utilize sialic acid as a sole carbon source. We demonstrate that the genes involved in the utilization of sialic acid are located within the *nan-nag* region of VPI-2 by complementation of *Escherichia coli* mutants and gene knockouts in *V. cholerae* N16961. We show that *nanH*, *dctP*, *nanA*, and *nanK* are highly expressed in *V. cholerae* grown on sialic acid. By using the infant mouse model of infection, we show that *V. cholerae* Δ*nanA* strain SAM1776 is defective in early intestinal colonization stages. In addition, SAM1776 shows a decrease in the competitive index in colonization-competition assays comparing the mutant strain with both O1 El Tor and classical strains. Our data indicate an important relationship between the catabolism of sialic acid and bacterial pathogenesis, stressing the relevance of the utilization of the resources found in the host's environment.

Cholera, a severe diarrheal disease, is caused by the gram-negative bacterium *Vibrio cholerae*, a natural inhabitant of brackish and estuarine waters (17, 18, 39). Only two serogroups, O1 and O139, are known to cause epidemic cholera, and only the O1 serogroup is associated with the seven pandemics of the disease (22, 40, 60). In 1992 a new non-O1 serovar emerged, designated O139 Bengal, causing cholera outbreaks in India and Bangladesh (16, 59). Between 1994 and 1996 El Tor O1 serogroups reemerged as the predominant cause of cholera on the Indian subcontinent; the O139 serogroup has remained relatively quiescent since, except for an outbreak in Dhaka, Bangladesh, in 2002 (1, 22). There are a number of virulence factors required for *V. cholerae* to cause the disease. These include cholera toxin, which is the main cause of the profuse diarrhea and is encoded within the CTX-phi phage, and the toxin-coregulated pilus (TCP), which is an essential colonization factor and is encoded within the *Vibrio* pathogenicity island (VPI) (also called the TCP island) (41, 43, 53, 70, 79). Another virulence factor of *V. cholerae* is neuraminidase (NanH), an extracellular enzyme that unmasks the receptors of cholera toxin and is also encoded within an integrative element named VPI-2 (24, 31, 34, 68).

VPI-2 is a 57-kb integrative element identified in *V. cholerae* O1 serogroup isolates (34, 35). The canonical VPI-2 region is present only in *V. cholerae* O1 strains, and the majority of the region has been deleted from O139 serogroup isolates (34). VPI-2 is inserted at a tRNA serine locus (VC1757.1) and is known to excise from the chromosome at a low rate to form a circular intermediate (48). VPI-2 encompasses loci VC1758 to VC1809, containing genes putatively involved in the transport and catabolism of sialic acid (*nan-nag* region), a restriction modification system, an integrase, and a Mu-phage like region (34). The *nanH* gene, encoding neuraminidase, is located directly downstream of the *nan-nag* region within VPI-2. NanH has the ability to cleave two sialic acid groups from the trisialogangliosides found in the intestinal mucus, releasing sialic acid into the environment (24, 69).

Sialic acids are a family of nine carbon ketosugars primarily found at terminal positions of numerous glycoconjugates on the surfaces of mammalian and avian cells, where they mediate a diverse range of cell-cell and cell-molecule interactions (61, 71, 75). Originally, it was thought that sialic acid was absent from prokaryotic cells (6). More recent data demonstrate that several bacterial pathogens, such as *Campylobacter jejuni*, enterohemorrhagic *Escherichia coli*, *Haemophilus influenzae*, *Haemophilus ducreyi*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pasteurella multocida*, and *Streptococcus agalactiae*, can put sialic acid residues on their cell surface (sialylate) as a method of masking the bacterial cell from the host immune system (8, 10, 12, 13, 26, 27, 29, 32, 37, 38, 44, 52, 58, 63, 66, 67, 73, 74, 78). These pathogens use different mechanisms to acquired sialic acid, which include de novo biosynthesis (*E. coli* and *N. meningitidis*), scavenging (*N. gonorrhoeae*), or precursor scavenging (*H. influenzae*) (52, 73, 74, 78). Pathogenic bacteria such as *Clostridium perfringens*, *E. coli* O157:H7, *H. influenzae*, and *P. multocida* also use sialic acid as a carbon and nitrogen source by scavenging it from the surrounding environment (15, 45, 62, 66, 67, 75). Evolutionary analysis of nearly 2,000 bacterial genome sequences found that the genes required for

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sialic acid catabolism are confined to commensal or pathogenic species. Specifically, most of these species colonize sialic acid-rich areas such as the gut and lungs, which suggests that sialic acid catabolism might play an important role in survival in vivo (3).

The catabolic pathway of sialic acid consists of three core enzymes (the Nan cluster): *N*-acetylneuraminic acid lyase, encoded by *nanA*, which cleaves sialic acid to release *N*-acetylmannosamine (NAM) and pyruvate; NAM kinase, encoded by *nanK*, which adds a phosphate group to NAM; and NAM-6-phosphate epimerase, encoded by *nanE*, which converts NAM-6-phosphate into *N*-acetylglucosamine-6-phosphate. Two additional enzymes, NagA and NagB, complete the catabolic pathway of sialic acid in bacteria (19, 45, 56, 75–77). The uptake of sialic acid can be performed by three well-characterized transporters: NanT, a single-component system, which belongs to the major facilitator superfamily; an ATP binding cassette (ABC) transporter; or a tripartite ATP-independent periplasmic C_4-dicarboxylate (TRAP) transport system, which consists of a periplasmic binding receptor (DctP) and two integral membrane proteins (DctM and DctQ) (57, 64, 75). To date, there is a lack of information regarding the possible role of sialic acid catabolism and pathogenicity (64). Also, the ability of *V. cholerae* to catabolize sialic acid has not been investigated yet. Here, we hypothesize that in the mucus-rich environment of the gut, *V. cholerae* strains with the ability to degrade sialic acid encounter a competitive advantage over those isolates that cannot utilize the aminosugar as a carbon source.

Toward that end, we demonstrate that *V. cholerae* can utilize sialic acid as a carbon and energy source. We determined that the genes involved in sialic acid catabolism are encoded within VPI-2 by using complementation analysis of *E. coli* mutants and knockout mutation analysis in *V. cholerae.* We found a significant increase in the expression of *nanH*, *nanA*, and *nanK*, as well as *dctP*, which is part of a putative TRAP transporter, and *rpiR*, a putative regulator, when sialic acid is utilized as a sole carbon source. We examined the infection dynamics of wild-type *V. cholerae* N16961 and SAM1776, a Δ*nanA* mutant strain, and demonstrate a significant growth disadvantage in the early stages of intestinal colonization for SAM1776. We performed competition assays using the infant mouse model and show that SAM1776 has a competitive index of 0.06 compared to the wild-type strain and over a fivefold decrease in the competitive index compared to an O1 classical strain. Overall, our data document, in vitro and in vivo, the importance of the ability of *V. cholerae* to utilize sialic acid as a carbon and energy source, suggesting a significant role in increasing survival and fitness in the host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. All genetic manipulations utilized *Escherichia coli* strains DH5α λpir, the β2155 λpir diaminopimelic acid (DAP) auxotroph, and S171-1 λpir. Unless otherwise stated, bacteria were grown on Luria-Bertani broth (LB) or M9 minimal medium (M9) at 37°C with aeration. The *E. coli* β2155 DAP auxotroph was cultured on medium containing 1 mM DAP (Fluka). The antibiotics added to LB had the following concentrations: streptomycin (Sm), 100 μg/ml; chloramphenicol (Cm), 25 μg/ml; and ampicillin, 25 μg/ml.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype	Reference
V. cholerae strains		
N16961	O1 El Tor, VPI-2$^+$, Sm^r	30
O395	O1 classical, VPI-2$^+$, Sm^r	46
MO10	O139, VPI-2$^-$, Sm^r	80
LAC1	O395, Δ*lacZ*, Sm^r	25
SAM2338	N16961, Δ*lacZ*, *gfp*, Sm^r	This study
SAM1776	N16961, ΔVC1776, Sm^r	This study
SAM1781	N16961, ΔVC1781, Sm^r	This study
SAM1782	N16961, ΔVC1782, Sm^r	This study
SAM1776C	SAM1776, pSAM1776C, Sm^r, Cm^r	This study
SAM1781C	SAM1781, pSAM1781C, Sm^r, Cm^r	This study
SAM1782C	SAM1782, pSAM1782C, Sm^r, Cm^r	This study
SAM1781C2	SAM1781, pSAM1781-82C, Sm^r, Cm^r	This study
E. coli strains		
BW25113	Wild-type strain	20
JW3194	BW25113, Δ*nanA*, Kn^r	9
JW3192	BW25113, Δ*nanE*, Kn^r	9
JW5538	BW25113, Δ*nanK*, Kn^r	9
JW3194C	JW3194, pSAM1776C, Kn^r, Cm^r	This study
JW3192C	JW3192, pSAM1781C, Kn^r, Cm^r	This study
JW5538C	JW5538, pSAM1782C, Kn^r, Cm^r	This study
JW3192C2	JW3192, pSAM1781-82C, Kn^r, Cm^r	This study
Plasmids		
pDS132	Suicide plasmid, Cm^r, SacB	55
pBBR1MCS	Expression plasmid, Cm^r	42
pJZ111	pCVD442, p*lac*::*gfp*::*lacZ*, Cm^r	J. Zhu
pSAM1776	ΔVC1776 cloned into pDS132	This study
pSAM1781	ΔVC1781 cloned into pDS132	This study
pSAM1782	ΔVC1782 cloned into pDS132	This study
pSAM1776C	VC1776 cloned into pBBR1MCS	This study
pSAM1781C	VC1781 cloned into pBBR1MCS	This study
pSAM1782C	VC1782 cloned into pBBR1MCS	This study
pSAM1781-82C	VC1781 and VC1782 cloned into pBBR1MCS	This study

Growth analysis. Precultures of either wild-type or mutant strains were grown to stationary phase at 37°C in LB, and 100 μl of these overnight cultures was added to 5 ml of fresh M9 minimal medium supplemented with *N*-acetylneuraminic acid (1 mg/ml) or D-glucose (1 mg/ml) as indicated (Sigma). Growth assays were done in triplicate on at least three occasions by adding 200 μl of the inoculated medium per well to a 96-well microtiter plate and incubating at 37°C under shaking conditions. Optical densities were measured at various time points using a Genios microplate reader and Magellan plate reader software (Tecan). Sigmaplot software was used to construct graphs based on the data obtained.

In silico analysis. We performed a BLAST search (blastp) against the sequenced genome of *V. cholerae* N16961 using as seeds the sequences of proteins known to be involved in the metabolism of sialic acid in other bacteria (4, 5, 30). The cutoff value for our search was 1e−20 except for those genes that were already annotated as the seed we were using.

RNA extraction and quantitative real-time PCR. Total RNA from *V. cholerae* N16961 was extracted at 1 h and 3 h postinoculation on M9 supplemented with sialic acid or glucose using RNAprotect Bacteria reagent (Qiagen) and an RNeasy mini kit (Qiagen); all samples were tested in triplicate. After confirmation of RNA quality, samples were DNase treated (DNA-free; Ambion) and then underwent a round of reverse transcription using SuperScript II reverse transcriptase (Invitrogen). The cDNA samples were diluted to a concentration of 4 ng/μl to perform quantitative PCR using SYBR green PCR Master Mix on an ABI Prism 7000 real-time PCR apparatus (Applied Biosystems); all assays were performed in triplicate at least twice. The gene-specific primers were designed using Primer3 software according to the real-time PCR guidelines and are listed in Table 2. The data were analyzed using ABI Prism 7000 SDS software (Applied Biosystems), and differences in the ratio of expression were extrapolated using the $\Delta\Delta C_T$ method (54). Plots were made using Sigma Plot.

TABLE 2. Oligonucleotides used in this study

Function and oligonucleotide	Sequence (5′→3′)	Annealing temp	Size (bp)
Splice overlap extension PCR			
VC1776A	GTTCGGTATTCGAGCGCAATCG	63	319
VC1776B	GTGTTGCGGCAGGTAAAGTTGC	63	
VC1776C	GCAACTTTACCTGCCGCAACACCATAAGCACCTTTCACTCC	63	368
VC1776D	ACCTAATGATTGGCATTCTTACCC	63	
VC1781A	CGTCGCAGCATTGACAGAAGC	61	332
VC1781B	TACAGGTTGTATGGAAACGACAG	61	
VC1781C	CTGTCGTTTCCATACAACCTGTAGCGGAAGGTCGATAC	61	318
VC1781D	CCTTCTGGCGTTACATAACCTG	61	
VC1782A	TGGCGGAAGGTCGATACAATAC	61	313
VC1782B	CCTTCTGGCGTTACATAACCTG	61	
VC1782C	CAGGTTATGTAACGCCAGAAGGGAATTGGTCTCGCAGAG	61	345
VC1782D	CAATACCCGCAAGAGTCATGTC	61	
Flanking			
VC1776FF	ATAGCGACCGACGATACTGG	57	2075
VC1776FR	CAACTGAAGCGGCTGCTGTT	57	
VC1781FF	GCACGCCAGTTGAACTTCTG	57	1878
VC1781FR	CGTTACCTGAAGCCATGGAC	57	
VC1782FF	AGCGTGACTTGCCTGATAGC	57	1853
VC1782FR	CCGCGACCGTTACAAGAACT	57	
Complements			
VC1776CF	CTCGAGACGGACAGTAGTTGAACTA	49	971
VC1776CR	GAGGCTCGATGAATATTCCTCCCTAG	49	
VC1781CF	CTCGAGGTTCATCAAGTCAGGAATTA	49	785
VC1781CR	GAGGCTCTGTTCCGCCGATATCGAT	49	
VC1782CF	CTCGAGCTCAATGGTTCAATAACGC	49	926
VC1782CR	GAGGCTCTTGCCTTTAATGCCATCGT	49	
Real-time PCR			
VC1775QF	GTAGAACCTGAGCTCGATATTG	62	152
VC1775QR	CGACCGACGATACTGGATGC	62	
VC1784QF	CGTCCATTGTAGCAAGTAGCGTAA	65	148
VC1784QR	TCGGTATCCCAAGTTATACCGCC	65	
VC1779QF	TGATGATCGTGCCATGCTTCAGC	65	123
VC1779QR	TTCGCGACATAAGGGAGCATGAC	65	
VC1776QF	AGGAGTGAAAGGTGCTTATGTCTG	65	114
VC1776QR	CATCTAACTTCCCATCAACGGCTT	65	
VC1782QF	AACAGGTTATGTAACGCCAGAAGG	65	128
VC1782QR	TGCATCATTAAGAATGGAGACTGGT	65	
VC1781QF	ACTGTCGTTTCCATACAACCTGTAA	65	124
VC1781QR	ACGTTATTCACACCTTCAATGCGC	65	
VC0328QF	ATCGAGCGTAACGTAGCGGTTGA	65	153
VC0328QR	AGTCAGGTTGTAGATGTCGATACC	65	

Complementation of *E. coli* JW3194, JW3192, and JW5538. *E. coli* JW3194, a Δ*nanA* mutant, was complemented with the *nanA* gene (VC1776) from *V. cholerae* N16961 amplified by PCR using primer pairs listed in Table 2 and cloned into pBBR1MCS using standard procedures, resulting in pSAM1776C (Table 2). The transformed *E. coli* JW3194/pSAM1776C strain (JW3194C) was grown on M9 medium supplemented with sialic acid. To complement *E. coli* JW3192, a Δ*nanE* mutant, and JW5538, a Δ*nanK* mutant, the *nanE* (VC1781) and *nanK* (VC1782) genes from *V. cholerae* N16961 were amplified from N16961 using

TABLE 3. Known gene products involved in sialic acid metabolism in bacteria and their homologues in *V. cholerae*

Homologue	Source[a]	Accession no.	*V. cholerae* locus	% Identity	E value	Function
NeuA	A	YP_854393	None			Neu5Ac CMP transferase
NeuB	A	YP_854394	None			Neu5Ac synthase
NeuC	A	YP_854392	None			UDP-GlcNAc 2-epimerase
Lst	B	BAA25316	None			α2,6-Sialyltransferase
LsgB	C	YP_249415	None			α2,3-Sialyltransferase
NanH		NP_231419	VC1784	100	0.00E+00	Neuraminidase
NanA	A	YP_858833	VC1776	29	5.00E−25	Neu5Ac aldolase
NanK	A	YP_858830	VC1782	40	8.00E−41	ManNAc kinase
NanE	A	YP_858831	VC1781	57	9.00E−50	ManNAc-6-P 2-epimerase
NagA	A	YP_851790	VC1783	33	1.00E−54	GlcNAc-6-P deacetylase
NagA	A	YP_851790	VC0994	61	1.00E−135	GlcNAc-6-P deacetylase
NagB	A	YP_851791	VCA1025	79	8.00E−119	GlcN-6-P deaminase
DctQ	C	YP_247714	VC1778	20	1.00E−06	TRAP transporter small permease
DctP	C	YP_247715	VC1779	48	6.00E−82	TRAP transporter periplasmic component
DctM	C	YP_247713	VC1777	37	3.00E−48	TRAP transporter large permease
NanT	A	YP_858832	None			MFS transporter
NanR	A	YP_858834	None			Sialic acid metabolism regulator
RpiR	D	YP_718908	VC1775	29	5.00E−26	Sialic acid metabolism regulator
NanM	A	AP_004802	VC1773	29	7E−46	Neu5Ac mutarotase
NanM	A	AP_004802	VC1774	35	6E−60	Neu5Ac mutarotase

[a] A, *E. coli* K-12 (65, 76); B, *Photobacterium damselae* JT0160 (82); C, *H. influenzae* NTHi 2019 (2); D, *H. somnus* 129PT (14).

primer pairs listed in Table 2 and cloned into these strains following the same procedure. Growth was measured as described in "Growth analysis" above.

Construction of *V. cholerae* N16961 Δ*nanA*, Δ*nanK*, Δ*nanE*, and Δ*lacZ* mutants. Using the *V. cholerae* N16961 genome sequence as a template, primers were designed to perform splice overlap extension PCR and obtain single-knockout mutants for VC1776 (*nanA*), VC1781 (*nanE*), and VC1782 (*nanK*). We constructed 267-bp, 354-bp, and 251-bp truncated versions of the three genes, respectively, as previously described (Table 2) (33, 48). Briefly, the Δ*nanA* construct was cloned into pDS132 to construct pSAM1776, which was then electroporated into *E. coli* DH5α and subsequently into *E. coli* β2155 λpir. *E. coli* β2155 λpir bearing pSAM1776 was cross-streaked with *V. cholerae* N16961 on LB plates supplemented with DAP to allow conjugation to occur. Exconjugants were replated on LB containing Sm and Cm. Single colonies were screened for single-crossover mutants using flanking primers and primers A and D from each of the genes (Table 2). Single-crossover mutants were inoculated on LB without antibiotics. Serial dilutions of the overnight culture were plated on LB–10% sucrose without NaCl and incubated at 30°C (47). Single colonies were screened by PCR, and products were purified and confirmed by sequencing. The same procedure was applied to construct *V. cholerae* SAM1781, a Δ*nanE* mutant, and SAM1782, a Δ*nanK* mutant. In order to complement *V. cholerae* SAM1776, pSAM1776C was electroporated into *E. coli* S17-1 λpir, and the cells were plated on LB containing Cm. The positive clones were cross-streaked with *V. cholerae* SAM1776 (Δ*nanA*). Cells with the plasmids were selected by plating on LB containing Sm and Cm. The same procedure was used for complementation of *V. cholerae* SAM1781 and SAM1782. SAM2338, a *lacZ* mutant of N16961, was constructed by using pJZ111 (a kind gift from J. Zhu), which exchanges *lacZ* for a gene encoding green fluorescent protein as described previously (11). Growth was measured as described in "Growth analysis" above.

Mouse infection and competition studies. Stationary-phase cells were prepared by overnight growth at 37°C for *V. cholerae* N16961, SAM2338, and SAM1776. *V. cholerae* LAC1, an O1 classical *lacZ*-negative strain, was incubated on LB broth at 30°C. For the in vitro competition assays, 1:1,000 dilutions from N16961, SAM1776, SAM2338, and LAC1 were mixed 1:1 according to their optical densities (SAM1776 and SAM2338, N16961 and LAC1, and SAM1776 and LAC1), and 100 μl was added to 5 ml of LB. The cultures were grown overnight at 37°C with aeration. Serial dilutions were plated on LB containing Sm and supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml; Fischer), and recovered CFU were counted for the in vitro competitive index (see below). The experiments were performed in triplicate at least twice. For the in vivo assays, 3- to 5-day-old CD-1 mice were orogastrically infected with *V. cholerae* as previously done by others (7). We used a total of six mice per group and performed the experiments at least twice. For the infection assay, CD-1 mice were inoculated with 50 μl of a 1:1,000 dilution of either N16961 or SAM1776. Mice were sacrificed at 1, 3, 6, 9, 12, and 24 h postinfection, and their small intestines were removed and homogenized and serial dilutions plated on LB containing Sm. Next, we performed in vivo competition assays. For these experiments, we infected each mouse using 50 μl of strain mixtures of SAM1776 and SAM2338, N16961 and LAC1, and SAM1776 and LAC1. We used a total of eight mice per group and performed the experiments at least twice. Mice were sacrificed at 24 h postinfection, and their small intestines were removed and homogenized and serial dilutions plated on LB containing Sm and supplemented with X-Gal (40 μg/ml; Fischer). Viable CFU were counted for the in vivo competitive index. The competitive indices were calculated as described by Osorio et al. (51).

RESULTS AND DISCUSSION

***V. cholerae* N16961 loci involved in sialic acid catabolism.** Initially sialic acid metabolism was thought to be confined to higher eukaryotes and metazoans. However, more recent data have shown that several bacterial human pathogens coat their surfaces with sialic acid to mask them from the host immune systems (75). Bacteria such as *Haemophilus influenzae*, *Neisseria meningitidis*, and *E. coli* K1 can either synthesize sialic acid de novo or scavenge sialic acid from the mammalian host (64, 75). We performed a BLAST search against the published genome of *V. cholerae* N16961 in order to identify putative homologues of previously characterized genes that are known to be involved in the metabolism of sialic acid in *Bacteria* (Table 3) (4, 5, 30). We did not find homologues of *neuC*, *neuB*, and *neuA* from *E. coli*, which are required for the synthesis of sialic acid and the addition of CMP to sialic acid before transfer by sialyltransferases to the bacterial cell surface (75). No homologues of the two known bacterial sialyltransferases (Lst and LsgB) was present within *V. cholerae* N16961 genome (Table 3) (23, 28, 82). However, a small portion at the 3′ end of Lst, between amino acids 498 and 664, showed high similarity with VC0727, a 236-amino-acid protein annotated as transcriptional regulator PhoU; the possible relationship between these two proteins remains to be elucidated (Table 3). As is well known, *V. cholerae* pathogenic isolates encode a neuraminidase (NanH), which unmasks the receptors of the cholera toxin (24, 31). NanH (VC1784) is located within VPI-2,

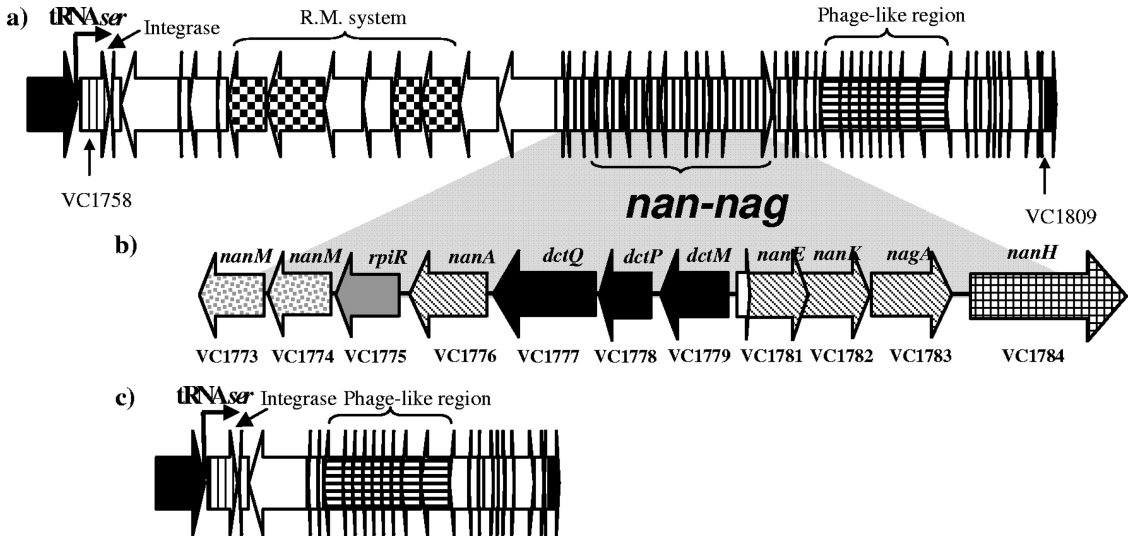

FIG. 1. Schematic diagram of VPI-2 and the *nan-nag* region in *V. cholerae* N16961. (a) VPI-2. Black arrows, core genes; bent black arrow, the $tRNA^{Ser}$ insertion site of VPI-2; thin vertically striped arrow, integrase; checkerboard arrow, restriction-modification system; bold vertically striped arrows, *nan-nag* region; horizontally striped arrows, phage-like region. (b) *nan-nag* region. Stippled arrows, mutarotases; light gray arrow, putative regulator; hatched arrows, putative genes involved in the catabolism of sialic acid; black arrows, putative genes involved in the transport of sialic acid; grid pattern arrow, neuraminidase. Above each arrow, the name of the putative gene is indicated. Below each arrow, the locus tag of the gene is indicated. (c) Structure of VPI-2 in *V. cholerae* MO10 (O139 strain). Black arrows, core genes; bent black arrow, the $tRNA^{Ser}$ insertion site of VPI-2; thin vertically striped arrow, integrase; horizontally striped arrows, phage-like region.

and adjacent to this gene are four genes encoding enzymes in the sialic acid catabolic pathway: *nanA* (VC1776), *nanK* (VC1782), *nanE* (VC1781), and *nagA* (VC1783) (Fig. 1, Table 3) (34, 35). Homologues of the NanT and NanR proteins from *E. coli*, which function as a sialic acid transporter and a regulator, respectively (56, 76, 77), were not identified. However, a gene for a putative TRAP transporter was identified in the BLAST search; this clustered with the catabolic genes VC1778 (*dctQ*), VC1779 (*dctP*), and VC1777 (*dctM*) and shared similarity to a sialic acid TRAP transporter from *H. influenzae* (2, 66, 67). In addition, a putative homologue of *rpiR* (VC1775), which in *H. influenzae* is a negative regulator of the sialic acid catabolism gene cluster, was present within the *nan-nag* region (75). Finally, two genes putatively encoding two sialic acid mutarotases, which are involved in the epimerization of α-*N*-acetylneuraminic acid into β-*N*-acetylneuraminic acid, were found adjacent to VC1775 (65) (Table 3).

In sum, we found that *V. cholerae* N16961 is genetically equipped to scavenge (*nanH*), epimerize (*nanM*), transport (*dctPQM*), and catabolize (*nanA*, *nanE*, *nanK*, and *nagAB*) sialic acid. Interestingly, *V. cholerae* is the only sequenced member of the family *Vibrionaceae* that does not contain the genes required for the synthesis of sialic acid and also is the only species that encodes a neuraminidase.

Growth of *V. cholerae* N16961, O395, and MO10 on sialic acid as a sole carbon and energy source. The in silico analysis indicates that *V. cholerae* N16961 carries the genes for the catabolism of sialic acid within VPI-2. Our next step was to determine whether *V. cholerae* was able to utilize sialic acid as a sole carbon and energy source. First we determined the ability of three *V. cholerae* strains to grow on M9 minimal medium supplemented with glucose or sialic acid (Fig. 2). *V. cholerae* N16961 (an O1 El Tor VPI-2-positive strain), O395 (an O1 classical VPI-2-positive strain), and MO10 (an O139 VPI-2-negative strain) all grew on M9 plus glucose, showing similar growth patterns and reaching final optical densities of between 0.33 and 0.4 (Fig. 2a). However, only N16961 and O395 grew on M9 plus sialic acid (Fig. 2b). This finding shows that *V. cholerae* VPI-2-positive strains are able to utilize sialic acid as a sole carbon and energy source, whereas the VPI-2-negative strain is not. Our data add to the limited list of bacterial pathogens and commensals that have been shown to utilize sialic acid as a carbon and energy source, such as *C. perfringens*, *E. coli* K-12, *E. coli* O157:H7, *Haemophilus influenzae*, and *Pasteurella multocida* (49, 66, 67, 76, 77).

Differential expression of the genes within the *nan-nag* region. The in silico BLAST search and in vitro growth assay strongly indicated that the genes involved in the degradation of sialic acid are carried within the *nan-nag* region of VPI-2. We examined whether the genes in the *nan-nag* region are expressed in the presence of glucose or sialic acid to determine if their expression was constitutive or induced. We investigated the expression of six genes, all within VPI-2: *rpiR*, encoding a putative regulator; *nanH*, encoding neuraminidase; *dctP*, encoding a putative transporter protein; and *nanA*, *nanK*, and *nanE*, encoding homologues of sialic acid catabolism proteins (Table 3). We isolated RNA from cultures of *V. cholerae* N16961 at 37°C with aeration on M9 supplemented with glucose or M9 supplemented with sialic acid. Real-time PCR was performed, and the six genes showed little expression on M9 supplemented with glucose at both 1 h and 3 h postinoculation compared to *rpoB* (Fig. 3). In contrast, N16961 cultured for 1 h and 3 h on M9 plus sialic acid showed markedly increased levels of expression of all six genes compared with the expression levels on M9 supplemented with glucose (Fig. 3). Two of the most noticeable differences in the expression ratios are

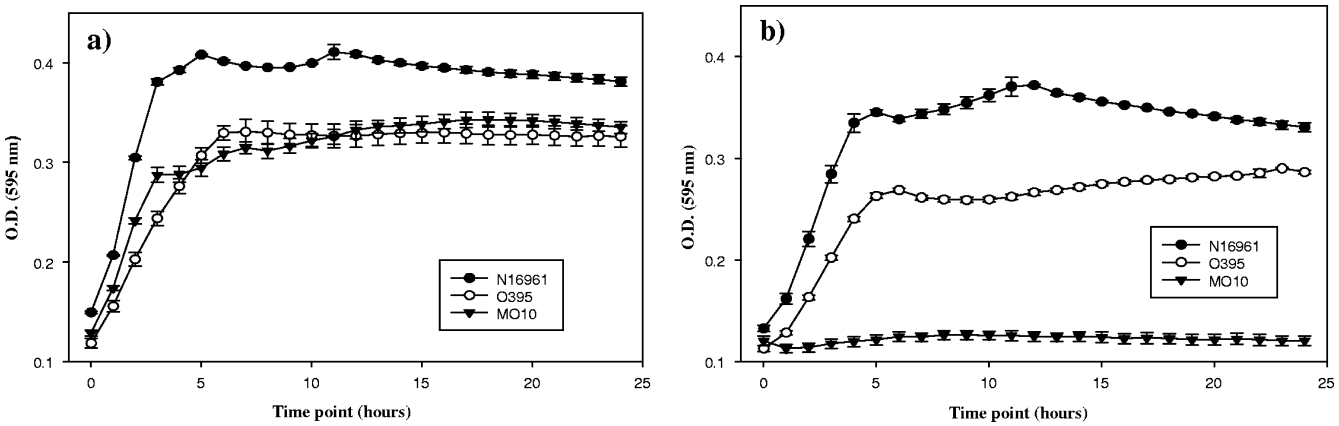

FIG. 2. Growth of different *V. cholerae* strains on minimal medium supplemented with glucose or sialic acid. The strains were incubated at 37°C under aerobic conditions on M9 minimal medium supplemented with glucose (a) or sialic acid (b). N16961 and O395 are VPI-2 positive, whereas MO10 is VPI-2 negative. Plots are represented on natural log scale. O.D., optical density. Error bars indicate standard deviations.

those of *dctP*, a component of the putative TRAP transporter, with over an 85-fold increase in expression on M9 supplemented with sialic acid compared to expression on M9 supplemented with glucose, and *nanA*, the first gene in the catabolic pathway, with a 150-fold difference in expression on M9 supplemented with sialic acid at 1 hour postinoculation compared to expression on M9 supplemented with glucose. Again, after 3 h of growth, the expression levels of both *dctP* and *nanA* on M9 supplemented with sialic acid are significantly higher than those on M9 supplemented with glucose, with 114-fold and 49-fold increases in their expression, respectively (Fig. 3). These results indicate that the levels of expression of the genes within the *nan-nag* region are strongly induced in the presence of sialic acid in *V. cholerae*. Interestingly, differential gene expression analysis of *V. cholerae* collected from the luminal fluid of the ligated ileal loop at 12 h postinoculation and from stationary-phase bacteria grown in LB broth also shows significantly increased expression of the *nan-nag* genes within the rabbit intestine (50). It was demonstrated in *Arthrobacter sialophilus* and *C. perfringens* that the expression of neuraminidase is increased by the presence of sialic acid, which is also seen in our study of *V. cholerae* (49, 81). It could be argued that sialic acid may act as a signaling molecule for entrance into the small intestine and that the putative TRAP transporter acts to transmit the signal from the host milieu to the bacterial cell interior.

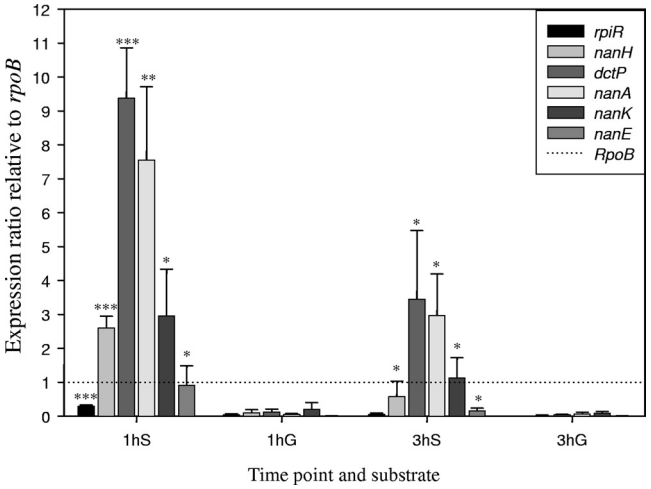

FIG. 3. Transcriptional analysis of the *nan-nag* genes. The bars represent expression ratio of the *nan-nag* genes relative to the expression of *rpoB* as determined using real-time PCR. *V. cholerae* N16961 was inoculated on M9 plus sialic acid (S) and M9 plus glucose (G). The RNA was extracted at 1 h and 3 h postinoculation. The genes under study were VC1775 (*rpiR*), VC1784 (*nanH*), VC1779 (*dctP*), VC1776 (*nanA*), VC1782 (*nanK*), and VC1781 (*nanE*). The expression of *rpoB* was used to normalize our test genes. The paired *t* test was used to infer statistical significance of the differences in expression of the genes between the two substrates. *, $0.01 < P \leq 0.1$; **, $0.001 < P \leq 0.01$; ***, $P \leq 0.001$. Error bars indicate standard deviations.

Complementation of *E. coli* JW3194, JW3192, and JW5538. To determine further the roles of *nanA* (VC1776), *nanE* (VC1781), and *nanK* (VC1782), we cloned each gene from *V. cholerae* N16961 into the broad-host-range vector pBBR1MCS (Table 1). We electroporated each plasmid into previously characterized *E. coli* deletion mutants for these genes (9). *E. coli* JW3194 (Δ*nanA*) was transformed with pSAM1776C, JW5538 (Δ*nanK*) with pSAM1782C, and JW3192 (Δ*nanE*) with pSAM1781C (Table 1) (9). First, we examined growth on LB and M9 supplemented with glucose as a sole carbon source (Table 4). *E. coli* JW3194, JW5538, and JW3192 showed growth patterns similar to that of strain BW25113 (Table 4). Subsequently, we studied their growth on M9 supplemented with sialic acid as a sole carbon source. As expected, BW25113 is able to utilize sialic acid as a sole carbon source and therefore grows on this medium (76). Both *nanA* (VC1776) and *nanK* (VC1782) from *V. cholerae* N16961 complemented the *E. coli* mutant strains JW3194 and JW5538, showing growth on M9 supplemented with sialic acid (Table 4). It is a note of interest that *E. coli* JW5538, the Δ*nanK* mutant, does not show a complete defect in growth compared to *E. coli* JW3194 (9, 20, 56, 76, 77). In addition, we were unable to complement *E. coli* JW3192, the Δ*nanE* mutant strain, with *nanE* (VC1781) from *V. cholerae*, and no growth was shown on M9 supplemented with sialic acid (Table 4). A total of three different VC1781 constructs were designed and tested in *E. coli* JW3192, but all

TABLE 4. Growth of *E. coli* and *V. cholerae* strains on different media

Species and strain	Growth on[a]:		
	LB	M9+G	M9+S
E. coli			
BW25113	***	**	**
JW3194	***	**	
JW3194C	***	**	**
JW3192	***	**	
JW3192C	***	**	
JW5538	***	**	*
JW5538C	***	**	**
JW3192C2	***	**	**
V. cholerae			
N16961	***	**	**
SAM1776	***	**	
SAM1776C	***	**	**
SAM1781	***	**	
SAM1781C	***	**	
SAM1782	***	**	*
SAM1782C	***	**	**
SAM1781C2	***	**	

[a] M9+G, M9 supplemented with glucose; M9+S, M9 supplemented with sialic acid; ***, final optical density of >0.8; **, final optical density of <0.8; *, lagged growth.

failed to complement (data not shown). This behavior is often shown when the mutation causes downstream effects in an adjacent gene; in this case the mutation in *nanE* might have affected *nanK*, which is likely due to overlapping of the two genes. In order to study whether the inability to complement *E. coli* JW3192 was due to a polar effect caused by the mutation, we cloned into pBBR1MCS the *nanE* and *nanK* operon using the primer pair VC1781F and VC1782R, generating pSAM1781-82C (Tables 1 and 2). In this case pSAM1781-82C complemented *E. coli* JW3192. Overall, our data indicate that the proteins encoded by VC1776, VC1781, and VC1782 have the same functions as NanA, NanE, and NanK in *E. coli*, respectively.

***V. cholerae* SAM1776 (Δ*nanA*), SAM1781 (Δ*nanE*), and SAM1782 (Δ*nanK*) mutant strains.** We constructed three isogenic knockout strains of *V. cholerae* N16961, with truncated versions of *nanA*, *nanE*, and *nanK* (Table 2). The three mutant strains SAM1776 (Δ*nanA*), SAM1781 (Δ*nanE*), and SAM1782 (Δ*nanK*) were inoculated into LB or M9 supplemented with glucose or sialic acid (Table 2; Fig. 4). All three strains grew similarly on LB and M9 supplemented with glucose (Table 4). However, neither SAM1776 nor SAM1781 grew on M9 supplemented with sialic acid, as expected (Fig. 4). *V. cholerae* SAM1782 (Δ*nanK*) showed impaired growth on M9 supplemented with sialic acid, similar to the growth pattern for *E. coli* JW5538 (Δ*nanK*), suggesting that both *E. coli* and *V. cholerae* have an additional kinase within the cell that can function similarly to NanK (Table 4).

Previous studies have shown that the TRAP transporter encoded by *dctPMQ* adjacent to the *nanA*, *nanE*, and *nanK* genes from *H. influenzae* is a high-affinity transporter for sialic acid and that this can rapidly increase the concentration inside the cell, causing a toxic effect (36, 76). To determine whether the presence of sialic acid impairs the growth of *V. cholerae* SAM1776, our Δ*nanA* mutant, due to internal accumulation, we compared the growth of the wild type and SAM1776 on LB or LB supplemented with 100 μM sialic acid and found no differences in growth on both media (data not shown). These data suggest that accumulation of sialic acid is not detrimental to the cell or that *V. cholerae* avoids accumulation of sialic acid in a yet-unknown manner.

Complementation of the *V. cholerae* SAM1776 and SAM1782 transformed with pSAM1776C and pSAM1782C, respectively, was shown by growth on M9 supplemented with sialic acid (Fig. 4). Surprisingly, we were unable to complement *V. cholerae* SAM1781 (ΔnanE) using several different constructs, including pSAM1781-82C (data not shown). It is possible that the regulation of the Nan cluster in *V. cholerae* is more complex than that in *E. coli* and requires the presence of certain regulatory sequences for optimal expression. Overall, our data show that VC1776, VC1781, and VC1782 are required for the catabolism of sialic acid.

Sialic acid catabolism in the infection and competition dynamics of *V. cholerae*. *V. cholerae* colonizes the heavily sialylated mucus of the human gut. The ability of *V. cholerae* to catabolize sialic acid as a carbon and energy source should give the organism a growth advantage compared to strains unable to utilize sialic acid. We performed single-infection assays in the infant mouse model to compare the wild type and Δ*nanA* mutant SAM1776. We inoculated, using the orogastric route, 3- to 5-day-old CD-1 mice with similar numbers of *V. cholerae* N16961 or SAM1776 organisms (~5×10^5). We inoculated six mice per group. We extracted the small intestines at 1, 3, 6, 9, 12, and 24 h postinfection and calculated the number of viable CFU for each time point for each strain (Fig. 5). For the wild-type strain, N16961, we found numbers equivalent to those shown by Angelichio et al. (7). At 1 hour postinoculation, both strains showed very similar numbers (~8×10^5); how-

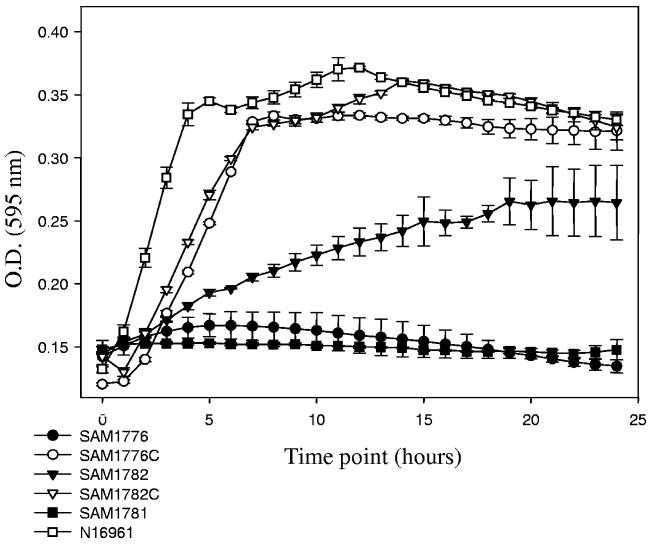

FIG. 4. Growth of *V. cholerae* sialic acid catabolism mutant strains. The constructed mutants and complements for the genes involved in the catabolism of sialic acid were incubated at 37°C under aerobic conditions. Growth was on M9 minimal medium supplemented with sialic acid. O.D., optical density. Error bars indicate standard deviations.

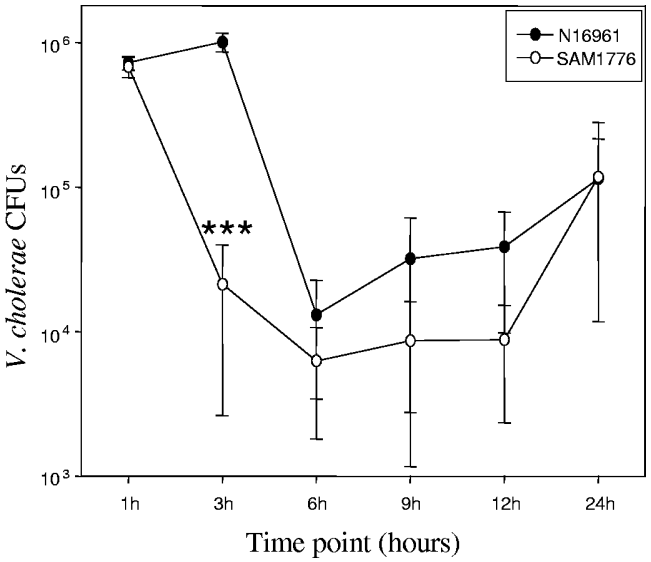

FIG. 5. Infection dynamics of *V. cholerae* N16961 and a sialic acid catabolism-deficient strain in the suckling mouse intestine. Three- to 5-day-old CD-1 mice were inoculated with *V. cholerae* N16961 or *V. cholerae* SAM1776 (Δ*nanA*). Six mice were used per strain and time point. The intestines were removed after 1, 3, 6, 9, 12, and 24 h. Serial dilutions of the homogenates were plated on LB containing Sm and the CFU counted. We used the paired *t* test to compare the numbers of CFU of N16961 and SAM1776 at the same time point. ***, $P < 0.0005$. Error bars indicate standard deviations.

ever, after 3 h, there was a noticeable decrease in the number of SAM1776 CFU compared to that at the 1-h time point and compared to the wild type (Fig. 5). After 6 h postinoculation, the wild-type N16961 did decrease in numbers between 4.5×10^3 and 2.3×10^4. The number of CFU recovered at 9 h postinoculation increased from the previous time point, with the number of *V. cholerae* N16961 cells ranging between 6.7×10^3 and 2.5×10^4 and the number of SAM1776 cells ranging between 1×10^2 and 1.7×10^4 (Fig. 5). No difference were found between the counts obtained after 9 and 12 h postinoculation (Fig. 5). Finally, at 24 h postinoculation, both strains achieved similar numbers of CFU. The data show that SAM1776 is unable to maintain the same high cell density as the wild-type strain in early stages of infection. This suggests that the ability to utilize sialic acid as a carbon source can increase the likelihood of successfully colonizing a heavily populated environment such as the human gut.

To test this further, we performed in vitro and in vivo competition assays between *V. cholerae* SAM2338 (N16961 Δ*lacZ*) and SAM1776 (Δ*nanA*). We also compared the competition indices of N16961 and SAM1776 with that of LAC1, a Δ*lacZ* derivative of O395, a classical strain, in order to see whether the inability of SAM1776 to utilize sialic acid had an effect in comparison to other *V. cholerae* strains rather than *V. cholerae* O1 El Tor. We inoculated orogastrically a total of eight CD-1 infant mice per group with mixtures of SAM1776 and SAM2338, N16961 and LAC1, or SAM1776 and LAC1. After overnight incubation, mice were sacrificed and intestinal numbers calculated. We found that the competitive index of *V. cholerae* SAM1776 versus SAM2338 was 0.06 (1/16.6), showing a 17-fold decrease; that of N16961 versus LAC1 was 0.17 (1/5.9), which is a 6-fold decrease; and that of SAM1776 versus LAC1 was 0.03 (1/33.3), indicating a 33-fold decrease. These data support the hypothesis that the ability of *V. cholerae* pathogenic strains to utilize sialic acid as a carbon source gives them a competitive advantage in the sialic acid-rich environment of the gut.

Conclusions. The diversity in the ways by which different bacterial pathogens utilize sialic acid as a means to increase their fitness is fascinating, ranging from avoiding phagocytosis or opsonization to preventing serum killing by impeding the insertion of the complement attack complex (64, 72, 75). However, to date, no direct relationship between sialic acid catabolism and bacterial pathogenesis has been established (64, 67, 73). Nonetheless, when studying carbon nutrition of commensal *E. coli* strains, Chang et al. found that a strain lacking the ability to take up and degrade sialic acid yielded fewer CFU in the feces than the wild type, strongly suggesting a role for sialic acid metabolism in bacterial colonization (15). However, Fabich et al. showed that a Δ*nanAT* strain of pathogenic *E. coli* EDL933 did not show the same disadvantage as the commensal strain (21). In this study, we show for the first time that sialic acid catabolism is important in the early infection stages of *V. cholerae*. The ability to utilize sialic acid as a carbon source confers a competitive advantage in the mouse intestine to strains of *V. cholerae* that carry a functional sialic acid gene cluster. The utilization of sialic acid as a carbon source might act as a "jump start" for *V. cholerae* when it is trying to colonize the intestine, where competition for resources is high.

The presence of the *nanA* gene, encoding the key enzyme in sialic acid degradation, has been documented for several bacterial pathogens, such as *H. influenzae*, *Mycoplasma* spp., *Salmonella enterica*, *Shigella boydii*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *V. vulnificus*, *Yersinia enterocolitica*, and *Yersinia pestis*, as well as for a wide group of human commensals, including the numerically important groups *Bacteroidetes* and clostridia (3). All these bacterial species colonize heavily sialylated mucous surfaces of the host, such as the lungs and the gut, suggesting that the ability to utilize this nine-carbon amino sugar may convey a fitness advantage. It is a note of interest that in most *V. cholerae* O139 serogroup strains, only open reading frames VC1758, VC1759, and VC1789 to VC1809 are present, whereas neuraminidase and the sialic acid transport and catabolism region are deleted from all O139 strains recovered after 1992 (34, 35). The O139 serogroup emerged in 1992 to cause numerous severe outbreaks of cholera in the Bengal delta region, surpassing El Tor isolates as the major cause of cholera in the region; however, by the mid-1990s the El Tor biotype reemerged as the predominant cause of cholera. We suspect that the deletion of VPI-2 from O139 isolates has a detrimental effect on the fitness of these strains. Among *V. cholerae* non-O1 serogroup isolates that cause gastroenteritis, neuraminidase and the sialic acid transport and catabolism region are present, indicating that this may be a significant determinant in their intestinal survival (48).

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