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Sialic Acid Catabolism and Transport Gene Clusters Are Lineage Specific in *Vibrio vulnificus*

Jean-Bernard Lubin, Joseph J. Kingston, Nityananda Chowdhury, and E. Fidelma Boyd

Department of Biological Sciences, University of Delaware, Newark, Delaware, USA

Sialic or nonulosonic acids are nine-carbon alpha ketosugars that are present in all vertebrate mucous membranes. Among bacteria, the ability to catabolize sialic acid as a carbon source is present mainly in pathogenic and commensal species of animals. Previously, it was shown that several *Vibrio* species carry homologues of the genes required for sialic acid transport and catabolism, which are genetically linked. In *Vibrio cholerae* on chromosome I, these genes are carried on the *Vibrio* pathogenicity island-2 region, which is confined to pathogenic isolates. We found that among the three sequenced *Vibrio vulnificus* clinical strains, these genes are present on chromosome II and are not associated with a pathogenicity island. To determine whether the sialic acid transport (SAT) and catabolism (SAC) region is universally present within *V. vulnificus*, we examined 67 natural isolates whose phylogenetic relationships are known. We found that the region was present predominantly among lineage I of *V. vulnificus*, which is comprised mainly of clinical isolates. We demonstrate that the isolates that contain this region can catabolize sialic acid as a sole carbon source. Two putative transporters are genetically linked to the region in *V. vulnificus*, the tripartite ATP-independent periplasmic (TRAP) transporter SiaPQM and a component of an ATP-binding cassette (ABC) transporter. We constructed an in-frame deletion mutation in *siaM*, a component of the TRAP transporter, and demonstrate that this transporter is essential for sialic acid uptake in this species. Expression analysis of the SAT and SAC genes indicates that sialic acid is an inducer of expression. Overall, our study demonstrates that the ability to catabolize and transport sialic acid is predominately lineage specific in *V. vulnificus* and that the TRAP transporter is essential for sialic acid uptake.

Sialic acids, also known as neuraminic or nonulosonic acids, are a family of nine-carbon alpha ketosugars. Sialic acids are widely distributed in deuterostomes, where they perform a number of functions such as cell-cell interactions, stabilizing glycoconjugates and cell membranes and acting as chemical messengers (46, 47). Sialic acids are utilized by commensal and pathogenic bacteria in a number of ways; for example, several pathogenic species of bacteria have been shown to decorate their cell surfaces with sialic acid to avoid recognition by the host immune system (46, 47, 49, 50). Bacteria can also utilize sialic acid as a sole carbon source, which was first shown in *Clostridium perfringens* (29). The enzymatic pathway to catabolize *N*-acetylneuraminic acid (Neu5Ac), the most common sialic acid, was shown by Vimr and colleagues in *Escherichia coli* to require three key enzymes (50, 51). First, Neu5Ac is broken down into *N*-acetylmannosamine (ManNAc) by a lyase/aldolase (NanA). ManNAc kinase (NanK) adds a phosphate group to generate *N*-acetylmannosamine-6-phosphate (ManNAc-6-P), upon which ManNAc-6-P epimerase (NanE) acts to convert it to *N*-acetylglucosamine-6-P (GlcNAc-6-P). In bacteria, the genes for the first three enzymes (NanA, NanK, and NanE) in the catabolism pathways are usually found clustered together in the genome (2). Recently, a novel epimerase was identified in *Bacteroides fragilis* and *Tannerella forsythia* that has no requirement for a phosphorylated substrate (9, 34). Finally, GlcNAc-6-P deacetylase (encoded by *nagA*) and glucosamine-6-P deaminase (encoded by *nagB*) then converts GlcNAc-6-P into fructose-6-P (Fru-6-P), which is a substrate in the glycolysis pathway.

Of the bacteria that contain a sialic acid catabolism (SAC) gene cluster, most are species known to colonize the animal intestine as pathogens or commensals (2, 9, 29, 40, 42, 51). In *Vibrio cholerae*, the causative agent of cholera, the SAC genes are present on chromosome I within the 57-kb *Vibrio* pathogenicity island-2 (VPI-2) region, which is confined to pathogenic strains (21, 22). By using an infant mouse model of infection, it was shown that the ability to catabolize sialic acid conveys a significant competitive advantage in the early stage of infection for *V. cholerae* (3). Genetically linked to the SAC cluster in *V. cholerae* are homologues of *siaPQM*, which encode a substrate-binding protein (SBP)-dependent secondary transporter belonging to the tripartite ATP-independent periplasmic (TRAP) transporter family (2, 3, 14, 27, 38). The homologous TRAP transporter associated with the SAC cluster in *Haemophilus influenzae* was shown to be highly efficient in the uptake of sialic acid (14, 27, 40). It has been more recently demonstrated in *V. cholerae* that the SBP of the TRAP transporter SiaPQM (VC1777 to VC1779) is a Na^+-dependent high-affinity secondary transporter for sialic acid also (28, 45).

There are at least four diverse families of solute transporters that are genetically linked with the SAC cluster among bacteria (2, 14, 27, 38, 39), the above-mentioned TRAP transporter from *V. cholerae* and *H. influenzae*, the major facilitator superfamily (MFS) NanT found in *Escherichia coli*, an ATP-binding cassette (ABC)-type transporter from *Haemophilus ducreyi*, and the sodium solute symporter (SSS) first identified in *Photobacterium profundum* (2, 14, 27, 38, 39). Among *Enterobacteriaceae*, NanT is the most prevalent transporter associated with the SAC cluster, whereas in the *Pasteurellaceae* and the *Vibrionaceae*, the TRAP transporter is the predominant type found (2, 14, 38, 39). Among

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Address correspondence to E. Fidelma Boyd, fboyd@udel.edu.

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the *Firmicutes*, the predominant transporters associated with the SAC cluster belong to the SSS, ABC, or sodium/proline (Sym) family of transporters (2, 14, 38, 39). Recent *in silico* and *in vitro* analyses of sialic acid transporters revealed the presence of two functional systems in *Salmonella enterica*, an MFS type and an SSS type (39).

Vibrio vulnificus is an inhabitant of the marine ecosystem and an opportunistic pathogen of humans, in whom it can cause severe and rapid septicemia (8, 15, 23, 31, 52). *Vibrio vulnificus* is commonly isolated from the water column and is also isolated in high numbers from oysters and other filter-feeding shellfish, and infections occur after the consumption of raw or improperly cooked shellfish (13) (15, 17, 23). Mortality associated with *V. vulnificus* infection is very high (>50%), making this bacterium the leading cause of death in the United States associated with the consumption of seafood (15, 17, 23, 43). A number of different typing schemes have been developed to separate *V. vulnificus* isolates into groups based on whether they are pathogenic or nonpathogenic by using biochemical, serological, or genetic methods (5–7, 10, 12, 13, 18, 26, 30, 33, 35, 36, 48). Thus, 3 biotypes are recognized among isolates based on phenotypic characteristics and host range criteria (7, 12, 25, 37). Based on 16S rRNA genotyping, Nilsson and workers found most environmental isolates had a distinct 16S rRNA genotype named genotype A and most clinical isolates had a distinct genotype designated genotype B (30). Phylogenetic analysis divides *V. vulnificus* strains into two major groupings designated lineage I and lineage II (7, 12, 25, 37). Lineage I is comprised almost entirely of strains that cause disease in humans and encompasses predominantly biotype 1 strains and are designated C-type strains in some typing schemes (33). Lineage II is comprised mainly of environmental or fish isolates encompassing biotypes 1 and 2 and are designated E-type strains (33). A third lineage is comprised of strains that are biotype 3 human pathogens (7, 12, 37). A recent *in vivo* study using a subcutaneously inoculated iron dextran-treated mouse model indicates that genotype is correlated with virulence of *V. vulnificus* biotype 1 strains (44).

Bioinformatic analysis demonstrated the presence of sialic acid catabolism and transporter gene clusters in the genome sequence of two *V. vulnificus* clinical isolates, YJ016 and CMCP6 (2). Using SOLiD sequencing analysis of four *V. vulnificus* strains, Gulig and colleagues demonstrated that three of these strains possessed genetically linked sialic acid catabolism and transport genes (16). In another study, it was demonstrated that a clinical strain of *V. vulnificus* had the ability to catabolize sialic acid, which was shown to be important for *in vivo* survival using a mouse model (20). Jeong and coworkers speculated that unlike *V. cholerae*, *V. vulnificus* has a NanT homologue for sialic acid transport (20).

In this study, we examined the genome arrangement of the SAC gene cluster within *V. vulnificus* and *Vibrio* species in general to determine the type of transporter associated with the cluster. Next, we examined a collection of *V. vulnificus* isolates, whose phylogenetic relationships are known, for the presence of the SAC gene cluster. To determine whether the presence of the SAC region is lineage specific, we mapped the distribution of *nanA*, which encodes aldolase, required in the first step of sialic acid catabolism, onto the phylogeny of the *V. vulnificus* isolates. Then, we investigated whether *V. vulnificus* isolates that encode the SAC region can catabolize sialic acid as a sole carbon source. Although sequenced *V. vulnificus* isolates appear to have two transporters associated with the SAC genes, the predominant transporter system found among *Vibrio* species is the TRAP system. We created a deletion mutation in TRAP system to examine whether it is essential for sialic acid transport in this species.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference
Strains		
Vibrio vulnificus		
YJ016	SAC^+ SAT^+; clinical isolate	11
CMCP6	SAC^+ SAT^+; clinical isolate	
JJK0731	CMCP6 Δ*siaM* (VV2_0731)	This study
Escherichia coli		
DH5α λ-pir	*pir*80d*lacZ*ΔM15 δ(*lacZYA-argF)U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	
β2155 DAP	Donor for bacterial conjugation; *thr1004 pro thi strA hsdS lacZ*ΔM15 (F *lacZ*ΔM15 *lacTRQJ36proAB*) *dapA* Erm^r *pirRP4* (Km^r from SM10)	
DH5α λ-pir ΔsiaM	DH5α λ-pir containing pDS132ΔsiaM	This study
β2155 DAP-ΔsiaM	β2155 harboring pDS132ΔsiaM	This study
Plasmids		
pDS132	Suicide plasmid, Cm^r, SacB	32
pDS132ΔsiaM	pDS132 harboring truncated *siaM*	This study

[a] SAC, sialic acid catabolism; SAT, sialic acid transport.

MATERIALS AND METHODS

Bacterial strains. Strains and plasmids used in this study are listed in Table 1. A total of 67 *V. vulnificus* natural isolates whose phylogenetic relationships are known were examined in this study (12). These isolates represent all three biotypes found in *V. vulnificus*. The isolates were collected between 1980 and 2005, from Asia, the United States, Europe, and India, with 27 isolates recovered from clinical sources and 40 from environmental (clams, mussels, fish, oysters, seawater, sediment) (12). All strains were grown aerobically (250 rpm) at 37°C in Luria-Bertani broth (Fisher Scientific, Fair Lawn, NJ) with a final NaCl concentration of 2% (Fisher Scientific) and stored at −80°C in LB broth with 20% (vol/vol) glycerol.

Molecular analysis. Chromosomal DNA was extracted from the 67 *V. vulnificus* isolates using the DNA isolation kit from Bio101 following the manufacturer's protocol (MP Biomedicals, Solon, OH). PCR primers for *nanA* were designed based on the sequence of *V. vulnificus* strain YJ016, VVA1199F-TTATCGCCGCTCCCCATACA and VVA1199R-GCAACG CCACCGTATTCAAC. PCR assays were performed in 25-μl reaction mixtures with a 2.5 μM concentration of each primer, 2.5 mM deoxynucleoside triphosphate (dNTP) mix, 10× PCR buffer, and 1 U of Choice *Taq* DNA polymerase (Denville Scientific, Metuchen, NJ). The PCR cycle program consisted of an initial denaturation step at 94°C for 1 min followed by 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles. PCR products were visualized on 1.0% agarose gels. A long-range PCR primer pair, VVA1194F and VVA1212R, was designed from *V. vulnificus* strain YJ016 to encompass an 18-kb region spanning the SAC and SAT gene clusters from open reading frames (ORFs) VVA1194 to VVA1212. Primer VVA1194F (TTG GTG TGC TAT CGG GTA CA) was designed within the *napC* gene, which encodes a periplasmic nitrate reductase, cytochrome *c*-type protein, and primer VVA1212R (AAA GGC ATC GCT CAC AAA CT), which encodes a preprotein translocase subunit, was designed within *secF*. The PCR assay was conducted using DyNAzyme EXT DNA polymerase (New England BioLabs, Ipswich, MA) in 50-μl reaction mixtures. The

TABLE 2 Primers used in this study

Primer name	Sequence (5′–3′)	T_m (°C)	Product size (bp)
SOE PCR primers			
SOEAsiaQF	GCtctagaGTGTTGTCCTTGGAAGCTGCG	57	527
SOEBsiaMR	ATGGCCAACCATGGATTTGGCA	57	
SOECsiaMF	TGCCAAATCCATGGTTGGCCAT GCATCGGCGGTCGGGATTGA	59	573
SOEDnanAR	CgagctcAACGATACTCAGAGCCCCCGT	57	
SOEFLsiaQF	CTCATTGGCTGTGCCATCGCT	58	1,998
SOEFLnanAR	CAAGGCCCGATCGCGGAAGT	60	1,224
RT-PCR primers			
nanA-QF	TTGGCTACTCTCAGCGCGCG	62	250
nanA-QR	TTGCCACTTCCGCGATCGGG	62	
nanE-QF	TCTTGCTTCGCGGATGGGCA	62	236
nanE-QR	CGTGTGATGGCCGAGCCCAC	63	
siaQ-QF	AGCCCGCCAAGGGGTAAACTG	62	247
siaQ-QR	TGTCGCTCATTGGCTGTGCCA	62	
mdh-QF	CCCGTTTCGATCAAAGGTTA	52	229
mdh-QR	CAATTGGCACAGTGGTGTTC	54	
PCR assay primers			
nanAF	TCGCGCATTTTCGCCACGAC		
nanER	GCGGCGAGTTGAGGCGTGTT	65	4,562
rpiRF	TACGCAAGCCCAGCGGCATG		
nanAR	TTGCCACTTCCGCGATCGGG	65	1,299
nanAF	TTGGCTACTCTCAGCGCGCG		
siaQR	TGTCGCTCATTGGCTGTGCCA	65	2,233
siaQF	AGCCCGCCAAGGGGTAAACTG		
nanER	CGTGTGATGGCCGAGCCCAC	65	2,309

program of an initial denaturation step at 94°C for 1 min followed by 94°C for 30 s, 60°C for 30 s, and 68°C for 12 min for 10 cycles, followed by 94°C for 30 s, 60°C for 30 s, 68°C for 18 min for 15 cycles, and then a final extension at 70°C for 5 min was used. The PCR products were visualized on 0.6% agarose gels. In addition, we performed four additional PCR assays encompassing *nanA* to *nanE*, *rpiR* to *nanA*, *nanA* to *siaQ*, and *siaQ* to *nanE* to test for the presence of the region within *nanA*-negative strains. PCR primer pairs were designed based on the sequence of *V. vulnificus* strain YJ016 and are shown in Table 2.

Bioinformatic analysis of sialic acid catabolism and transporter genes among *Vibrionaceae*. We performed BLAST searches (BLASTP) against the sequenced genome database (4). We used as probes the sequences of proteins encoded by *nanA* (aldolase), *nanE* (epimerase), and *siaP* (periplasmic binding component of the TRAP transporter) from *V. vulnificus* YJ016. In addition, we examined the genes immediately upstream and downstream of the region containing *siaPQM* and *nanA*, *nanEK*, and *nagA* among all sequenced *Vibrionaceae* to investigate whether additional transporter genes were present.

Growth analysis in minimal medium supplemented with sialic acid. Two strains positive for the presence of *nanA*, YJ016 and CMCP6, and three strains negative for the presence of *nanA*, C7184, ss108A-3A, and 98-640 DP B9, were examined for their ability to grow in sialic acid as a sole carbon source. Precultures of each strain were grown to stationary phase at 37°C in LB, and a 100-μl aliquot of these 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) (Sigma-Aldrich, St. Louis, MO), of which a 200-μl aliquot per well was added to a 96-well microtiter plate and incubated at 37°C with shaking. Optical density at 595 nm (OD_{595}) was measured hourly for 24 h using a Genios microplate reader and Magellan plate reader software (Tecan US, Durham, NC). Graphpad Prism software was used to construct graphs based on the data obtained. Growth assays were performed in triplicate at least two times.

Mutant construction. An in-frame nonpolar deletion mutant was constructed using the splicing by overlap extension (SOE) PCR and allelic exchange procedure (19). We used the *V. vulnificus* CMCP6 genome sequence as a template to design primers, which were purchased from Integrated DNA Technologies (Coralville, IA), to perform SOE PCR and obtain an in-frame single knockout mutation for VV2_0731, which contains the *siaM* gene (Table 2). A 774-bp deletion was created in VV2_0731, resulting in a 510-bp nonpolar truncated version of the *siaM* gene (1,284 bp), thus creating a nonfunctioning TRAP transporter. Briefly, the *siaM* AD PCR fragment was cloned into the suicide vector pDS132 (32), which was designated pDSΔsiaMAD and electroporated into *Escherichia coli* strain DH5α λ-pir. pDSΔsiaMAD was then plasmid purified and transformed into the *E. coli* strain β2155, a diaminopimelic acid (DAP) auxotroph, and pDSΔsiaMAD was then conjugated into *V. vulnificus* CMCP6 via cross-streaking on LB plates containing 0.3 mM DAP (Sigma-Aldrich, St. Louis, MO). Growth from these plates was then transferred to LB 2% NaCl plates containing chloramphenicol (25 μg/ml) to select for *V. vulnificus* pDSΔsiaMAD only. Exconjugate colonies were cultured overnight in the absence of antibiotics, and serial dilutions were plated on LB 2% NaCl containing 10% sucrose to select for cells that had lost pDSsiaMAD. Double-crossover deletion mutants were then screened by PCR using the SOEFLsiaQF and SOEFLnanAR primers and confirmed by sequencing.

cDNA synthesis and RT-PCR. Prior to RNA isolation, *V. vulnificus* CMPC6 was cultured overnight in LB containing 2% NaCl at pH 7 and diluted in fresh M9 minimal medium containing 2% NaCl at pH 7 supplemented with 1 mg/ml of sialic acid or glucose and grown to an OD_{595} of 0.6 (log phase). Total RNA was extracted from *V. vulnificus* CMPC6 using RNAprotect bacterial reagent (Qiagen, Valencia, CA) and an RNeasy minikit (Qiagen) according to the manufacturer's protocols. RNA quantity was measured on a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA), and samples were then treated with DNase to remove genomic DNA (Turbo DNase, Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. One microgram of each sample of RNA was assessed on a 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer (Mediatech Inc., Herndon, VA) to ensure the quality of the samples. cDNA was synthesized by using SuperScript II reverse transcriptase (RT; Invitrogen) according to the manufacturer's protocol, with 500 ng of RNA as the template, and primed by 200 ng of random hexamers. cDNA samples were diluted 1:25 and 1:125 and used as templates for semiquantitative reverse transcription-PCRs using gene-specific primers designed using Primer3 software and listed in Table 2.

RESULTS AND DISCUSSION

***Vibrio vulnificus* SAC and SAT region is predominately lineage I specific.** We performed a BLAST search against the three genomes of *V. vulnificus* strains YJ016, CMCP6, and MO6-24/O in the NCBI genome database to identify homologues of sialic acid catabolism and transport genes from *V. cholerae* (1–3). We identified the genes encoding enzymes in the sialic acid catabolic and transport pathways, *nanA* (VV2_0730), *nanK* (VV2_0735), *nanE* (VV2_0734), *nagA* (VV2_0736), and *siaPQM* (VV2_0731 to VV2_0733), in strain CMCP6 (Fig. 1). The sequences of these genes among the three *V. vulnificus* strains YJ016, CMCP6, and MO6-24/O were >99% identical, suggesting a highly conserved region. The order and arrangement of the sialic acid catabolism (SAC) and transport (SAT) genes in all three strains of *V. vulnificus* were identical to those present in *V. cholerae* (Fig. 1). However, in *V. vulnificus*, the SAC and SAT region was carried on chromosome II and was not associated with a pathogenicity island (Fig. 1). An additional difference between the two species is the absence of the *nanH* gene from *V. vulnificus* strains. In *V. cholerae*, the *nanH* gene encodes sialidase (neuraminidase), a glycohydrolase that cleaves sialic acid from high-order gangliosides, releasing free sialic acid.

The SiaM protein (VV2_0731) shared an overall sequence

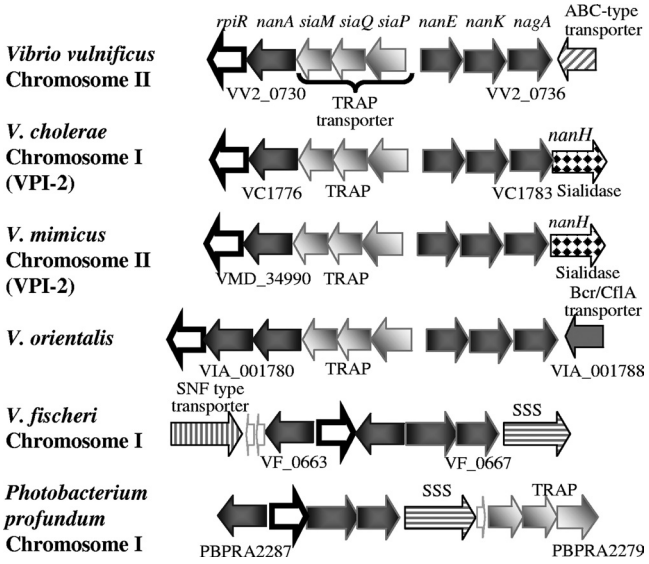

FIG 1 Genome context and arrangement of sialic acid catabolism (SAC) and transporter (SAT) gene clusters among *Vibrio* species. Open reading frames (ORFs) are indicated as arrows, the direction of which shows the direction of transcription. Numbers underneath ORFs represent locus tags. ORFs of similar shading represent homologous genes among the different species examined. The following annotated ORFs are shown: *rpiR*, transcriptional regulator; *nanA*, *N*-acetylneuraminic acid aldolase/lyase; *siaPQM*, TRAP transporter; *nanE*, *N*-acetylmannosamine-6-P epimerase; *nanK*, *N*-acetylmannosamine kinase; *nagA*, *N*-acetylglucosamine-6-phosphate deacetylase; ABC, ATP-binding cassette transporter; SSS, sodium solute symporter transporter.

identity of 95% with *V. cholerae* SiaM (VC1777) and 57% with SiaM from *Haemophilus influenzae*, which was shown previously to be part of a high-affinity, Na(+)-dependent unidirectional secondary TRAP transporter for sialic acid (40). No homolog of NanT, an MFS transporter present in *E. coli*, was identified in any of the genomes examined. Directly downstream of *nagA* in all three sequences was a homologue of an ABC-type transporter component (Fig. 1). However, this periplasmic ABC component has domains associated with oligopeptide binding and not amino sugar or carbohydrate binding in general. It appears that *V. vulnificus* is genetically capable of sialic acid transport into the bacterial cell, which can then be catabolized as a carbon source.

Vibrio vulnificus strains YJ016, CMCP6, and MO6-24/O are clinical isolates from Asia. We wanted to determine whether the SAC and SAT region is confined to clinical isolates of *V. vulnificus*, similar to *V. cholerae.* To accomplish this, we examined a collection of 67 natural isolates, which is comprised of both clinical and environmental isolates, for the presence of *nanA*. The *ana* gene encodes aldolase required in the first step of sialic acid catabolism. Of the 27 clinical isolates examined for the presence of *nanA*, 21 were positive for the gene by our PCR assay, whereas of the 40 environmental isolates examined, 17 strains gave a positive PCR band, indicating that they contained *nanA*. To further investigate the distribution of *nanA* among *V. vulnificus* isolates, we mapped the presence or absence of *nanA* onto the phylogeny of these strains (Fig. 2). The phylogenetic tree was constructed based on the analysis of six housekeeping genes as previously described (12) and contained two major lineages: lineage I was comprised primarily of clinical strains and lineage II was comprised predominately of environmental strains (12). We found that 34 out of the 37 lineage I isolates were positive for the presence of *nanA*, whereas only 7 of the 26 lineage II isolates were positive for the presence of *nanA*. These data demonstrate a strong correlation between the presence of the SAC region and lineage I strains (Fig. 2). The three strains in lineage I that lacked *nanA* were clinical strains isolated in Japan and the United States, and they did not cluster together on the tree, suggesting independent loss of the region. To determine whether the entire SAC and SAT region is missing in these strains and if a similar deletion event occurred in each strain, we used a long-range PCR assay with a primer pair that encompassed the SAC and SAT gene clusters (Fig. 3A). We designed the primer pair within genes that are conserved among other *Vibrio* species (Fig. 3B). We examined four *nanA*-negative strains from divergent branches of the *V. vulnificus* phylogenetic tree. An expected 18-kb PCR band was obtained for YJ016, which contain the SAT and SAC gene clusters. Only an ~7-kb-sized PCR product was obtained for three of the *nanA*-negative strains, JY1701, E86, and L-180, which demonstrated that the SAC and SAT region was absent from these strains and that the same deletion event occurred (Fig. 3D). Since no product was obtained for K2637, we speculate that a larger deletion event that involved either *vva1194* or *vva1212* or both genes occurred. We performed four additional PCR assays on these strains to amplify regions within the SAT and SAC clusters encompassing *nanA* to *nanE*, *rpiR* to *nanA*, *nanA* to *siaQ*, and *siaQ* to *nanE*. Only the positive control strain YJ016 gave a positive PCR band in these assays, indicating that these regions are absent from *nanA*-negative strains (Fig. 3E and F).

***Vibrio vulnificus* SAC- and SAT-positive strains can utilize sialic acid as a sole carbon and energy source.** *In silico* analysis showed that *V. vulnificus* lineage I isolates carry the genes required for the transport and catabolism of sialic acid. Our next step was to determine whether *V. vulnificus* is capable of growth on sialic acid as a sole carbon and energy source. We examined two *nanA*-positive isolates and three *nanA*-negative isolates for their ability to grow in M9 minimal medium supplemented with glucose (M9 plus glucose) or sialic acid (M9 plus sialic acid) (Fig. 4). All *V. vulnificus* strains grew in M9 plus glucose, showing similar growth patterns and reaching final OD_{595} values of between 0.44 and 0.5 (Fig. 4A). However, only *V. vulnificus nanA*-positive strains grew in M9 plus sialic acid, whereas the *nanA*-negative strains failed to do so (Fig. 4B). This finding demonstrates that *V. vulnificus nanA*-positive strains are able to take up and utilize sialic acid as a sole carbon and energy source. Our data add to the growing list of bacterial species that have been shown to utilize sialic acid as a carbon and energy source (2, 3, 9, 20, 34, 38–40, 42).

***Vibrio vulnificus* SiaPQM (VV2_0731 to VV2_0733) TRAP transporter is essential for growth on sialic acid as a sole carbon source.** Bioinformatic analysis of sialic acid catabolism genes among *Vibrionaceae* indicates that the TRAP transporter system is the predominant type of transporter genetically linked to the catabolism genes within this group (Table 3). However, closer examination of the genes flanking SAC identified several species whose DNA encoded two types of transporters genetically linked to the catabolism genes (Fig. 1). In *Vibrio orientalis*, both a TRAP system and a Bcr/CflA transporter were adjacent to the sialic acid catabolism genes (Fig. 1 and Table 3). In *P. profundum* strain SS9, both a TRAP and an SSS system were genetically linked to the SAC genes. In *V. vulnificus*, the TRAP *siaPQM* operon is linked to the

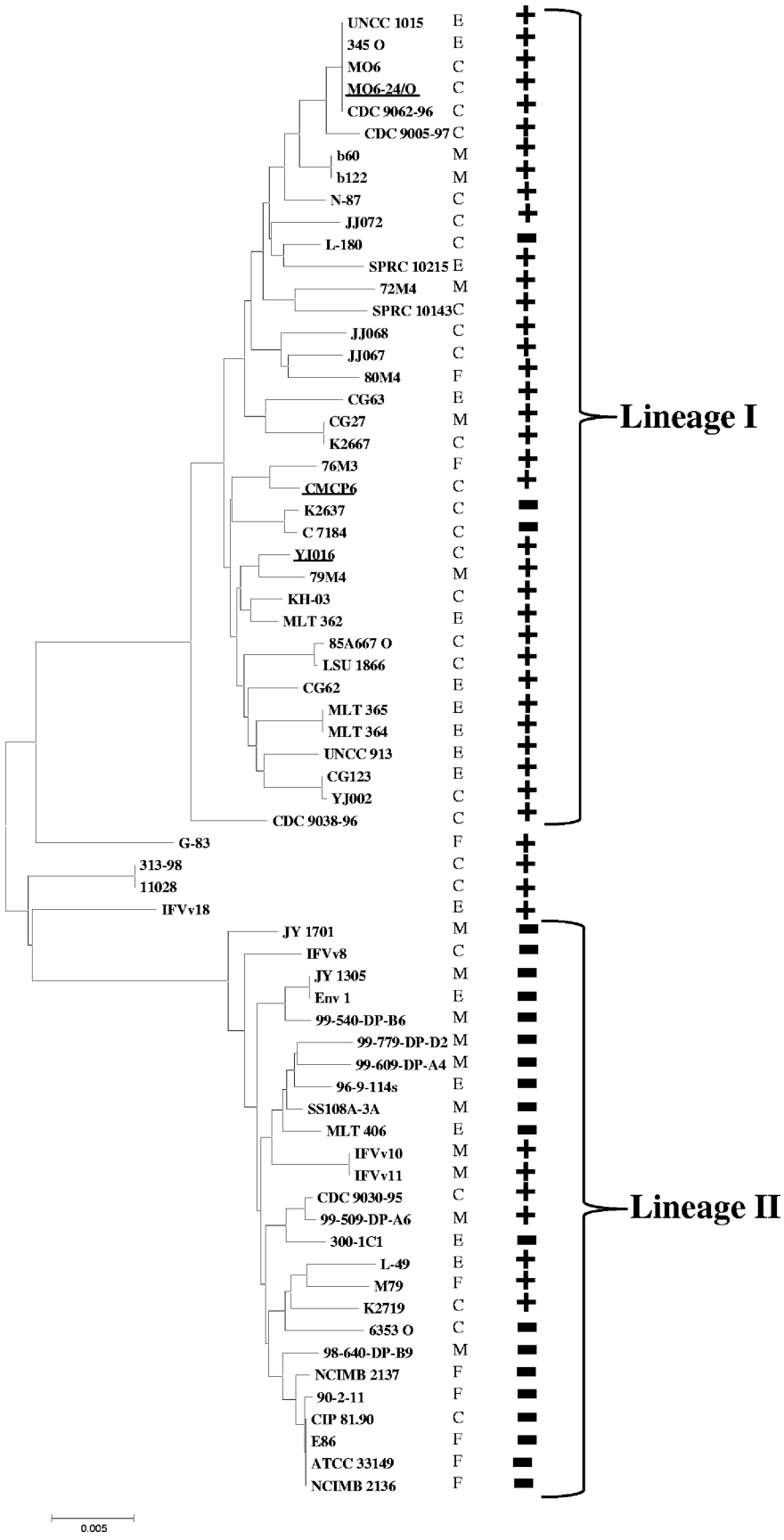

FIG 2 Distribution of *nanA* within the *V. vulnificus* phylogeny. The phylogenetic tree of *V. vulnificus* is based on the analysis of six housekeeping genes using the Kimura method and constructed using the neighbor-joining method as previously described (12). PCR assays were performed using *nanA*-specific primers and genomic DNA of *V. vulnificus* strains as templates. Positive and negative PCR results are indicated by "+"and "−," respectively. The three sequenced *V. vulnificus* strains are underlined. The source of each isolate is also shown where C is for clinical, E for environmental, F for fish, and M for mollusk.

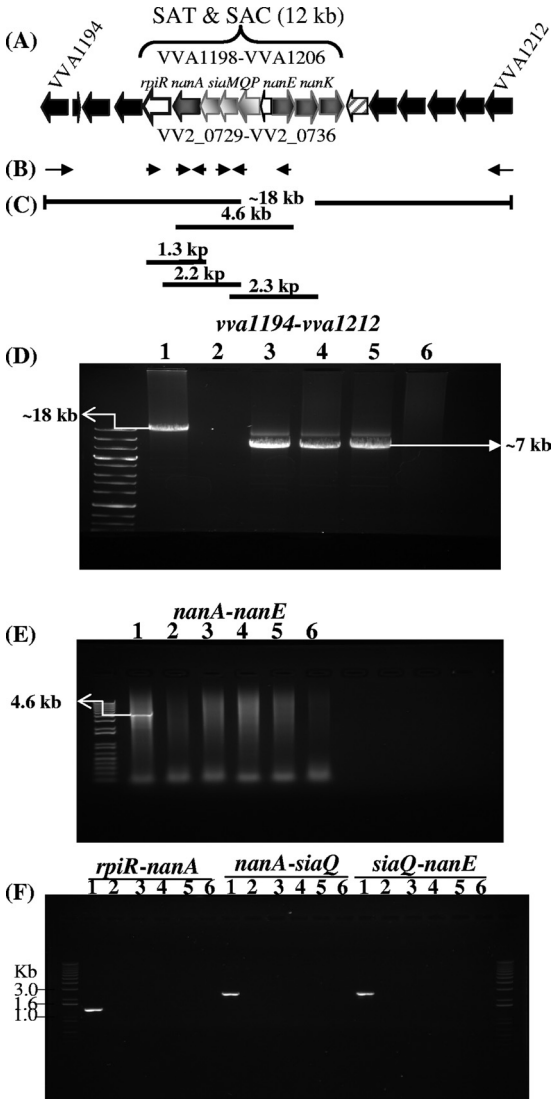

FIG 3 PCR analysis of *V. vulnificus nanA*-negative strains. (A) Schematic of region examined. Solid arrows represent ORFs and black filled arrows represent ORFs outside the SAT and SAC region. Numbers above and below ORFs represent locus tags for strains YJ016 and CMCP6, respectively. (B) Line arrows indicate locations of primers used in PCR assays. (C) Black solid lines indicate regions amplified by primer pairs. (D) Long-range PCR assay. The lane to the left of lane 1 shows a 1 kb plus DNA ladder (Invitrogen). Lanes: 1, *nanA*-positive strain YJ016; 2 to 5, *nanA*-negative strains K2637, JY1701, E86, and L-180; 6, negative control, no template. A product of ~18 kb was obtained from YJ016, whereas an ~7-kb band was obtained from all other strains except K2637. (E) *nanA*-to-*nanE* PCR assay. Lanes 1 to 6 are as in panel D. A product of 4.6 kb was obtained from YJ016 only. (F) PCR assays for *rpiR-nanA*, *nanA-siaQ*, and *siaQ-nanE*. Lanes 1 to 6 are as in panel D. PCR products were obtained from YJ016 only.

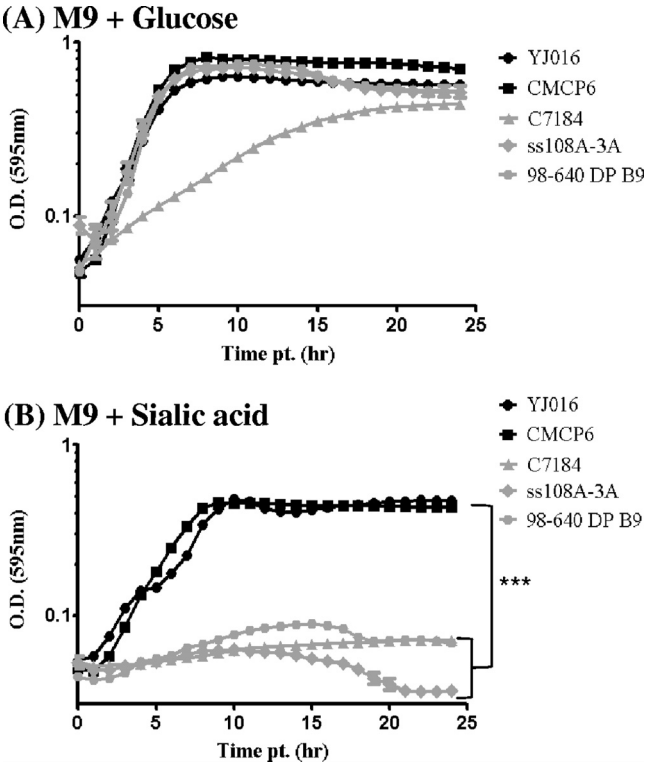

FIG 4 Growth analysis of *V. vulnificus* in M9 minimal medium supplemented with glucose (A) or *N*-acetylneuraminic acid (sialic acid) (B) as a sole carbon source. Two *V. vulnificus* strains that carry the sialic acid catabolism and transporter gene clusters (YJ016 and CMCP6) and three *nanA*-negative strains (C7184, ss108A-3A, and 98-640 DP B9) were examined in minimal medium supplemented with glucose (A) or sialic acid (B). All cultures were grown in triplicate, and each experiment was performed at least twice using two biological replicates. Plots are represented on a natural log scale. OD, optical density. Error bars indicate standard deviations. An unpaired Student *t* test was used to determine statistically significant difference between cells grown in glucose and cells grown in *N*-acetylneuraminic acid. ***, $P < 0.001$.

catabolism genes as well as an ABC transporter component. Thus, we investigated whether the TRAP system was essential for sialic acid uptake in this species. We constructed an isogenic knockout strain of *V. vulnificus* CMCP6 with an in-frame nonpolar truncated version of *siaM*. The *siaM* gene encodes a large permease containing 12 transmembrane helices, which is an essential component of the TRAP transporter system. The SiaPQM TRAP system was first characterized in *H. influenzae* and has recently been shown in *V. cholerae in vitro* experiments to be a high-affinity sialic acid transporter (28, 40). Wild-type CMCP6 and mutant strain JJK0731 were inoculated into LB or M9 supplemented with glucose, *N*-acetylglucosamine (NAG), or *N*-acetylneuraminic acid (sialic acid) as the sole carbon source. Both wild-type and mutant strains demonstrated similar growth patterns in LB (data not shown), indicating that there is not a general growth defect in these strains. In addition, the wild type and the mutant strain JJK0731 grew similarly in M9 supplemented with glucose (Fig. 5A). However, strain JJK0731 did not grow in M9 supplemented with sialic acid as a sole carbon source, whereas the wild-type strain showed growth when examined under the same growth conditions (Fig. 5B). Thus, these data demonstrate that SiaPQM is essential for growth on sialic acid as the sole carbon source in *V. vulnificus*. As expected, both the wild-type and mutant strains grew similarly in M9 supplemented with NAG, which is one of the products of the sialic acid catabolism pathway and does not require SiaPQM for uptake into the bacterial cell (Fig. 5A).

A recent study in *V. cholerae* proposed that a different TRAP transporter unrelated to SiaPQM from *V. cholerae* and *V. vulnificus* was required for sialic acid transport (41). Sharma and colleagues proposed that the VC1929 ORF, which encodes a C4-

TABLE 3 Distribution of sialic acid transport and catabolism genes among the sequenced *Vibrionaceae*

Species strain(s)	Gene[c]	
	Transport	Catabolism
Vibrio vulnificus lineage I[a]	TRAP	NanAEK
V. vulnificus lineage II[b]	X	X
V. cholerae pathogenic	TRAP	NanAEK
Vibrio cholerae nonpathogenic	X	X
Vibrio mimicus VM603/573/MB451	TRAP	NanAEK
Vibrio parahaemolyticus	X	X
V. parahaemolyticus 16	TRAP	NanAEK
Vibrio fischeri ES114/MJ1	SSS	NanAEK
Vibrio shilonii AK1	TRAP	NanAEK
Vibrio sinaloensis DSM21326	TRAP	NanAEK
Vibrio orientalis CIP10289	TRAP/Bcr/CflA NanAEK	
Vibrio sp. strain MED222	TRAP	NanAEK
Vibrio anguillarum	TRAP	NanAEK
Photobacterium profundum SS9/3TCK	TRAP/SSS	NanAEK
P. damselae CIP102761	NK	NanAEK
Aliivibrio salmonicida	NK	NanA only

[a] Three lineage I strains lacked the SAC and SAT clusters.
[b] Seven lineage II strains contained the SAC and SAT gene clusters.
[c] Abbreviations: SSS, sodium solute symporter; TRAP, tripartite ATP-independent periplasmic; NK, not known; X, absent.

dicarboxylate-binding periplasmic protein named DctP, was part of a TRAP system (VC1927 to VC1929) involved in sialic acid transport (41). They argued that in an El Tor strain of *V. cholerae*, VC1929 was a mannose-sensitive hemagglutinin that was required for sialic acid utilization (41). This was an unexpected finding since VC1929 shared high sequence homology with C4-dicarboxylate permeases that are involved in the transport of malate, fumurate, or succinate (45). Recently, we investigated the role of DctP (VC1929) in *V. cholerae* using its nonpolar knockout mutant and demonstrated that it plays a role in C4-dicarboxylate but not sialic acid uptake (our unpublished data). A homologue of VC1929, VV1_0030, which shows 89% amino acid identity to DctP, is also present in *V. vulnificus*. However, like *V. cholerae*, DctP (VV1_0030) in *V. vulnificus* does not appear to be involved in sialic acid transport given that our *siaM* (VV2_0731) mutant is no longer able to utilize sialic acid as a sole carbon source.

Sialic acid induces expression of SAC and SAT genes. Next, we examined whether the catabolism and transporter genes are constitutively expressed or induced in the presence of sialic acid. We examined the expression of three genes: *siaQ*, *nanA*, and *nanE*. The *siaQ* gene encodes a small permease containing 4 transmembrane helices, which is a component of the TRAP transporter, *nanA* encodes *N*-acetylneuramic acid aldolase, the first enzyme in the sialic acid catabolism pathway, and *nanE* encodes ManNAc-6-P epimerase, which catalyzes the last step in the pathway. We isolated RNA from cultures of *V. vulnificus* CMCP6 grown at 37°C with aeration in M9 supplemented with glucose or sialic acid. Semiquantitative reverse transcriptase PCR (RT-PCR) was performed, and the three genes showed no expression in M9 supplemented with glucose at 3 h postinoculation (Fig. 6). However, RT-PCR analysis of *V. vulnificus* strain CMCP6 cultured in M9 supplemented with sialic acid showed that all three genes were expressed (Fig. 6). Our results demonstrate that the level of expression of both the transporter and catabolism genes is induced in the presence of sialic acid in *V. vulnificus*. This is in agreement with what has been shown for *V. cholerae*, where both the catabolism and transporter genes were highly expressed in the presence of sialic acid (3). Kim and colleagues have recently demonstrated that the divergently transcribed *siaP* and *nanE* genes are under the control of the negative regulator *rpiR* (VV2_0730) in *V. vulnificus* (24). They showed that both the catabolic and transport genes are induced in the presence of sialic acid. They also found that *N*-acetylmannosamine-6-phosphate specifically bound to RpiR (NanR) and functioned as the inducer of the *nan* genes (*nanEKnagA* and *siaPQM*) (24).

Overall, our data demonstrate that the ability to catabolize and transport sialic acid is predominately lineage specific in *V. vulnificus*, and clinical isolates are capable of growth in sialic acid as a sole carbon and energy source. In addition, we have demonstrated that the *siaPQM* genes (VV2_0731 to VV2_0733) genetically linked to the catabolism genes encode a TRAP transporter for sialic acid uptake. *Vibrio vulnificus* is an opportunistic pathogen and has two modes of entry, either through the gastrointestinal tract or through wound infection. It is unlikely that there is one key virulence factor that is essential for virulence at both of these

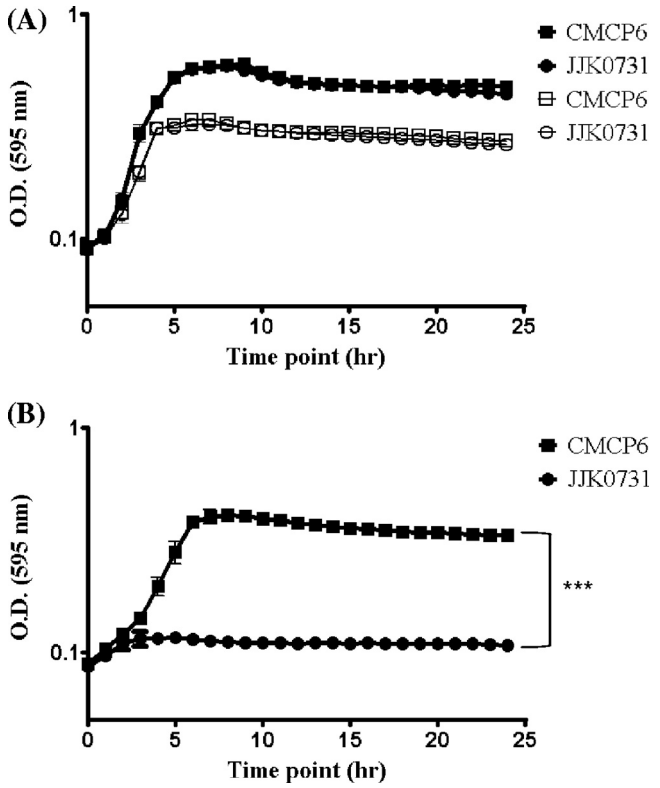

FIG 5 Growth analysis of *V. vulnificus* wild-type strain CMCP6 and its nonpolar *siaM* deletion mutant strain JJK0731 in M9 minimal medium supplemented with glucose or *N*-acetylglucosamine (NAG) (A) or *N*-acetylneuraminic acid (sialic acid) (B). (A) Upper two lines with symbols of solid squares and circles indicate the growth pattern in M9 plus glucose, and the lower two are of M9 plus NAG. Plots are represented on natural log scale. OD, optical density. All cultures were grown in triplicate, and each experiment was performed at least twice using two biological replicates. Error bars indicate standard deviation. An unpaired Student *t* test was used to determine statistical difference between mutant strain cells and wild-type strain cells grown in *N*-acetylneuraminic acid (sialic acid). ***, $P < 0.001$.

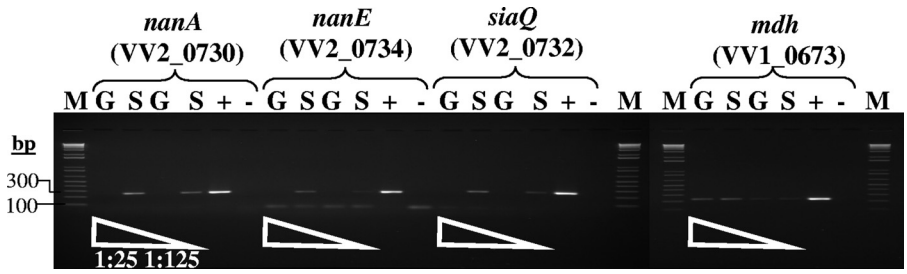

FIG 6 Expression analysis of sialic acid catabolism (e.g., *nanA*, *nanE*) and transporter (e.g., *siaQ*) genes, and a reference (housekeeping) gene (*mdh*) in *V. vulnificus* strain CMCP6 in the presence of glucose (G) or sialic acid (S). cDNA samples were diluted 1:25 and 1:125 and used as templates for semiquantitative RT-PCRs. Genomic DNA of CMCP6 and a PCR mixture without any DNA were used as positive (+) and negative (−) controls, respectively. The values on the left are the molecular mass (bp) standard of 1 kb plus DNA ladder (Invitrogen).

sites of infection. Thus, the loss of the SAC genes in some lineage I isolates may make them less competitive in the human gut, but they may still be formidable wound pathogens. The linking of sialic acid catabolism genes with a high-affinity sialic acid transport system would certainly be advantageous to a species either in environments where nutrients are limited or in environments where competition for nutrients is high, such as the animal gut. The presence of free glucose is highly limited in the animal intestine, and gastrointestinal pathogens have evolved to take advantage of alternative carbon sources in this niche. Mucous membranes are ubiquitous within the intestinal tract and are made up of mucins, which are sialylated glycoproteins and represent a potential nutrient source. Many different pathogenic and commensal species possess sialic acid catabolism genes that enable them to utilize glycosaminoglycans (GAGs) as carbon and nitrogen sources. Sialic acids are nine-carbon amino sugars that are present at the termini of GAGs in many cell types. Commensals and pathogens can carry the gene for sialidases that cleave terminal sialic acids, releasing them for uptake into the bacterial cells. Thus, the ability to take up and utilize sialic acid as a sole carbon source should be advantageous to gastrointestinal pathogens. Indeed, it has been demonstrated that in both *V. cholerae* and *V. vulnificus*, the ability to use sialic acid as a sole carbon source increases their fitness *in vivo* (3, 20).

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