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, DE 19716, USA

Corresponding author
E. Fidelma Boyd – fboyd@udel.edu

Department of Biological Sciences, 341 Wolf Hall, University of Delaware
Newark, DE 19716

Phone: (302) 831-1088. Fax: (302) 831-2281
Sialic or nonulosonic acids are nine carbon alpha-keto sugars that are present in all vertebrate mucous membranes. Among bacteria, the ability to catabolize sialic acid as a carbon source is present mainly in pathogen 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 that are genetically linked. In V. 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 V. vulnificus sequenced clinical strains that 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, a tripartite ATP-independent periplasmic (TRAP) transporter SiaPQM and a component of an 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.
Introduction

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-acetamannosamine (ManNAc) and phosphoenolpyruvate (PEP) by a lyase/aldolase (NanA). ManNAc kinase (NanK) adds a phosphate group to generate N-acetylmannosamine-6-phosphate (ManNAc-6-P), which ManNAc-6-P epimerase (NanE) acts on to convert into 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 encode 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 *V. 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
By using an infant mouse model of infection, it was shown that the ability to catabolize sialic acid confers 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 *H. influenzae* was shown to be highly efficient in the uptake of sialic acid (14, 27, 40). And it has been more recently demonstrated in *V. cholerae* that the SBP of the TRAP transporter SiaPQM (VC1777-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 *E. coli*, an ABC-type transporter from *H. 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 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 where 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 \textit{V. vulnificus} isolates into groups based on whether they are pathogenic or non-pathogenic by using biochemical, serological, or genetic methods (5-7, 10, 12, 13, 18, 26, 33, 35, 36, 30, 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 \textit{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 (53). Lineage II is comprised mainly of environmental or fish isolates encompassing biotypes 1 and 2 and are designated as E-type strains (53). A third lineage is comprised of strains that are biotype 3 human pathogens (7, 12, 37). A recent \textit{in vivo} study using a subcutaneously inoculated iron dextran-treated mouse model indicates that genotype is correlated with virulence of \textit{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 \textit{V. vulnificus} clinical isolates YJ016 and CMCP6 (2). Using SOLiD sequencing analysis of four \textit{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 \textit{V. vulnificus} had the ability to catabolize sialic acid, which was shown to be important for \textit{in vivo} survival using a mouse model (20). Jeong and co-workers speculated that unlike \textit{V. cholerae}, \textit{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 *V. vulnificus* sequenced 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 transporter in this species.

**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 1980-2005, from Asia, USA, 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% (v/v) glycerol.

**Molecular analysis.** Chromosomal DNA was extracted from the 67 *V. vulnificus* isolates using the DNA isolation kit from Bio101 following the manufactures protocol (MP Biomedicals, Solon, OH). PCR primers for *nanA* were designed based on the sequence of *V.*
**V. vulnificus** strain YJ016, VVA1199F- TTATCGCCGCTCCCCATACA and VVA1199R-
GCAACGCCACCGTATTCAAC. PCR assays were performed in 25μl reactions with 2.5μM
concentration of each primer, 2.5mM dNTP mix, 10x PCR buffer, and 1U of Choice™ Taq
DNA polymerase (Denville Scientific, Metuchen, NJ, USA). The PCR cycle program consisted
of an initial denaturation step at 94°C for 1 min followed by 94°C for 30s, 55°C for 30s, 72°C
for 1 min for 30 cycles. PCR products were visualized on 1.0% agarose gels. 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 ORFs VVA1194 to
VVA1212. Primer VVA1194F (TTG GTG TGT CGG GTA CA) was designed within the
napC gene that encodes a periplasmic nitrate reductase, cytochrome C-type protein and
primer VVA1212R (AAA GGC ATC GCT CAC AAA CT) was designed within secF that
encodes a preprotein translocase subunit. The PCR assay was conducted using DyNAzyme™
EXT DNA polymerase (New England Biolabs, Ipswich, MA, USA), in 50 μl reactions. The
program of an initial denaturation step at 94 °C for one minute followed by 94 °C for 30s, 60
°C for 30s, and 68 °C for 12 min for 10 cycles, followed by 94 °C for 30s, 60 °C for 30s, 68 °C
for 18 min for 15 cycles, 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 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 encoding siaPQM and nanA, nanEK and nagA among all sequenced Vibrionaceae to investigate whether additional transporter genes were present.

**Growth analysis in minimal media 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 media supplemented with N-acetylneuraminic acid (1 mg/ml) or D-glucose (1 mg/ml) (Sigma Aldrich, St. Louis, MO) of which a 200µl aliquot per well was added to a 96-well microtiter plate and incubated at 37°C with shaking. Optical densities at 595 nm (O.D.595) were measured hourly for 24 h using a Genios microplate reader and Magellan plate reader software (TECAN US, Durham, NC, USA). 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 non-polar deletion mutant was constructed using the splicing by overlap extension (SOE) PCR and allelic exchange procedure (19). We used 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 encodes the siaM gene (Table 2). A 774-bp deletion was created in VV2_0731 resulting in a 510 bp non-polar truncated version of the siaM gene (1284 bp), thus creating a non-functioning TRAP transporter. Briefly, the siaM AD PCR fragment was cloned into the suicide vector pDS132 (32), which was designated as pDSSiaMAD and electroporated into the Escherichia coli strain DH5α λ-pir. pDSSiaMAD was then plasmid purified and transformed into the E. coli strain β2155, a diaminopimelic acid (DAP) auxotroph, and pDSSiaMAD 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* pDSsiaMAD 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 reverse transcriptase PCR (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 media 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 Bacteria reagent (Qiagen, Valencia, CA) and an Rneasy mini kit (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) as per the manufacturer’s protocol. One μg of each sample of RNA was assessed on a 1% agarose gel in 1× TBE buffer (Mediatech Inc, Herndon, VA) to ensure quality of the samples. cDNA was synthesized by using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol, with 500 ng of RNA as template and primed by 200 ng of random hexamers. cDNA samples were diluted 1:25 and 1:125 and used as templates for semi quantitative reverse transcription-PCR reactions using gene-specific primers designed using Primer3 software and are listed in Table 2.
**RESULTS AND DISCUSSION**

*Vibrio vulnificus* sialic acid catabolism (SAC) and transport (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-VV2_0733) in strain CMCP6 (Fig. 1). The sequences of these genes among the three *V. vulnificus* strains YJ016, CMCP6 and MO6-24/O was >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* was 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 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, a 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 catabolised 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 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 which was comprised primarily of clinical strains and lineage II which 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 USA 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 size 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 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 3F).

*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 media supplemented with glucose (M9+glucose) or sialic acid (M9+sialic acid) (Fig. 4). All *V. vulnificus* strains grew in M9+glucose, showing similar growth patterns, and reaching final O.D.595 values between 0.44 and 0.5 (Fig. 4A). However, only *V. vulnificus* nanA-positive strains grew in M9+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 uptake and utilize sialic acid as a sole carbon and energy source. Our data adds 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-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 *V. 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 a SSS system were genetically linked to the SAC genes. In *V. vulnificus*, the TRAP siaPQM operon is linked to the 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 non-polar 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 characterised in *H. influenzae* and has recently been shown in *V. cholerae* in vitro experiments to be a high affinity sialic acid transporter (28, 40). The 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 sole carbon sources. 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 ORF VC1929, which encodes a C4-dicarboxylate-binding periplasmic protein named DctP, was part of a TRAP system (VC1927-VC1929) involved in sialic acid transport (41). They argued that in an El Tor strain of V. cholerae, VC1929 was a mannose-sensitive haemagglutinin 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, fumarate, or succinate (45). Recently, we investigated the role of DctP (VC1929) in V. cholerae using its non-polar knock-out mutant and demonstrated that it plays a role in C4-dicarboxylate but not sialic acid uptake (Chowdhury et al, unpublished data). A homologue of VC1929 is also present in V. vulnificus, VV1_0030, which shows 89% amino acid identity to DctP. 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, nanoA, and nanoE. The siaQ gene encodes a small permease containing 4 transmembrane helices, which is a component of the TRAP transporter, nanoA encodes N-acetylneuramic acid aldolase, the first enzyme in the sialic acid catabolism pathway and nanoE encodes ManNAc6-P epimerase that 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. Semi-quantitative reverse
transcriptase PCR (RT-PCR) was performed and the three genes showed no expression in M9 supplemented with glucose at 3 hr post-inoculation (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 (*nanEK*na**gA* and *siaPQM*) (24).

Overall our data demonstrates 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_0731-0733) 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 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 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 uptake and utilize sialic acid as a sole
carbon source should be advantageous to gastrointestinal pathogens. Indeed, it has been
demonstrated that in both \textit{V. cholerae} and \textit{V. vulnificus} the ability to use sialic acid as a sole
carbon source increases their fitness \textit{in vivo} (3, 20).

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References


Table 1. Bacterial strains and plasmids used in this study.

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<td>This study</td>
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<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α λ-pir</td>
<td>pir80dlacZM15(lacZYA-argF)U169,recA1,hsdR17, deoRthi-IsupE44 gyrA96 relA1</td>
<td></td>
</tr>
<tr>
<td>β2155 DAP</td>
<td>Donor for bacterial conjugation; thr1004 pro thi strA hsdS lacZ_</td>
<td></td>
</tr>
<tr>
<td>M15 (F lacZ_M15 lacTRQ1_36 proA_proB) dapA Ermr pirRP4</td>
<td>(KmR from SM10)</td>
<td></td>
</tr>
<tr>
<td>DH5α λ-pir ΔsiaM</td>
<td>DH5α λ-pir containing pDS132ΔsiaM</td>
<td>This study</td>
</tr>
<tr>
<td>β2155 DAP-ΔsiaM</td>
<td>β2155 harboring pDS132ΔsiaM</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pDS132</td>
<td>Suicide plasmid, Cm⁹, SacB</td>
<td>(32)</td>
</tr>
<tr>
<td>pDS132ΔsiaM</td>
<td>pDS132 harboring truncated siaM</td>
<td>This study</td>
</tr>
</tbody>
</table>

SAC = Sialic acid catabolism, SAT = Sialic acid transport
Table 2. Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′-3′)</th>
<th>Tm (°C)</th>
<th>Product size (bp)</th>
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</thead>
<tbody>
<tr>
<td><strong>Splice overlap extension PCR:</strong></td>
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<tr>
<td>SOEAsiaQF</td>
<td>GCTCTAGAGTGTTGGTCCTTGGGAAGCTGCG</td>
<td>57</td>
<td>527</td>
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<tr>
<td>SOEBsiaMR</td>
<td>ATGGCCAACCATGGATTTGGCA</td>
<td>57</td>
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</tr>
<tr>
<td>SOECsiaMF</td>
<td>TGCCAAAATCCATGGTTGGCCAT</td>
<td>59</td>
<td>573</td>
</tr>
<tr>
<td></td>
<td>GCATCGGCGCGTGGGATTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOEDnanAR</td>
<td>CGAGCTCAACGATACGAGCGCCGT</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>SOEFLsiaQF</td>
<td>CTCATTGGGCTGGCCATCGCT</td>
<td>58</td>
<td>1998</td>
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<tr>
<td>SOEFLnanAR</td>
<td>CAAGGCCCGATCGCGGAAGT</td>
<td>60</td>
<td>1224</td>
</tr>
<tr>
<td><strong>RT-PCR:</strong></td>
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<td></td>
</tr>
<tr>
<td>nanA-QF</td>
<td>TTGGCTACTCTCAAGCAGCCG</td>
<td>62</td>
<td>250</td>
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<tr>
<td>nanA-QR</td>
<td>TTGGCCACTTCCCGGATCGGG</td>
<td>62</td>
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<tr>
<td>nanE-QF</td>
<td>TCTTGCTTCCGGATGGGCA</td>
<td>62</td>
<td>236</td>
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<tr>
<td>nanE-QR</td>
<td>CGTGCGATGGCCGAGCCAC</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>siaQ-QF</td>
<td>AGCCCGCCAGGGGTAAAAGT</td>
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<td>247</td>
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<tr>
<td>siaQ-QR</td>
<td>TGTCGCTTCATTGGCTGTGCA</td>
<td>62</td>
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<tr>
<td>mdh-QF</td>
<td>CCGTCTTCGATCAAAGTGA</td>
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<td>229</td>
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<tr>
<td>mdh-QR</td>
<td>CAAATGCGACAGGTGTGTT</td>
<td>54</td>
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<tr>
<td><strong>PCR assays</strong></td>
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</tr>
<tr>
<td>nanAF</td>
<td>TCGCGCATTTCGCCAGCAC</td>
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<td></td>
</tr>
<tr>
<td>nanER</td>
<td>GCGGCCGATGGAGCGGTGTT</td>
<td>65</td>
<td>4562</td>
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<tr>
<td>rpiRF</td>
<td>TACGCAAGGCCAGCGGCGT</td>
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<td></td>
</tr>
<tr>
<td>nanAR</td>
<td>TTGCCACTTCCCGGATCGGG</td>
<td>65</td>
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<tr>
<td>nanAF</td>
<td>TGTCGACTTCCAGCCGCG</td>
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<tr>
<td>siaQR</td>
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<tr>
<td>siaQF</td>
<td>AGCCCGCCAGGGGTAAAGT</td>
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<td></td>
</tr>
<tr>
<td>nanER</td>
<td>CGTGCGATGGCGAGGCCAC</td>
<td>65</td>
<td>2309</td>
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</table>
Table 3. Distribution of sialic acid transport and catabolism genes among the sequenced *Vibrionaceae*

<table>
<thead>
<tr>
<th>Species strain(s)</th>
<th>Transport</th>
<th>Catabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio vulnificus</em> lineage I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>V. vulnificus</em> lineage II&lt;sup&gt;b&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>V. cholerae</em> pathogenic</td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em> non-pathogenic</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Vibrio mimicus</em> VM603/573/MB451</td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em> 16</td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Vibrio fischeri</em> ES114/MJ1</td>
<td>SSS</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Vibrio shilonii</em> AK1</td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Vibrio sinaloensis</em> DSM21326</td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Vibrio orientalis</em> CIP10289</td>
<td>TRAP/Bcr/CflA NanAEK</td>
<td></td>
</tr>
<tr>
<td><em>Vibrio sp</em> MED222</td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em></td>
<td>TRAP</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Photobacterium profundum</em> SS9/3TCK</td>
<td>TRAP/SSS</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>P. damsela</em> CIP102761</td>
<td>NK</td>
<td>NanAEK</td>
</tr>
<tr>
<td><em>Aliivibrio salmonicida</em></td>
<td>NK</td>
<td>NanA only</td>
</tr>
</tbody>
</table>

SSS = Sodium Solute Symporter, TRAP = Tripartite ATP-independent periplasmic, NK = not known, X = absent, a = 3 lineage I strains lacked the SAC and SAT clusters, and b = 7 lineage II strains contained the SAC and SAT gene clusters.
FIGURE LEGENDS

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 color 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.

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 denotes 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 of SAT and SAC region. Numbers above and below ORFs represent locus tags for strains YJ016 and CMCP6, respectively. (B). Line arrows indicate location of primers used in PCR assays. (C). Black solid lines indicate regions amplified by primer pairs. (D). Long-range PCR assay. Lane 1 kb plus DNA ladder (Invitrogen), Lane 1: nanA-positive strain YJ016,
Fig. 4. Growth analysis of *V. vulnificus* in M9 minimal media supplemented with (A) glucose or, (B) N-acetylneuraminic acid (sialic acid) as a sole carbon source. Two *V. vulnificus* strains YJ016 and CMCP6 that carry the sialic acid catabolism and transporter gene clusters and three *nanA*-negative strains (C7184, ss108A-3A and 98-640 DP B9) were examined in minimal media supplemented with (A) glucose or (B) sialic acid. All cultures were grown in triplicate and each experiment was performed at least twice using two biological replicates. Plots are represented on natural log scale. O.D., optical density. Error bars indicate standard deviation. An unpaired Student’s t-test was used to determine statistical difference between cells grown in glucose and cells grown N-acetylneuraminic acid. ***, *p* < 0.001.

Fig. 5. Growth analysis of *V. vulnificus* wild-type strain CMCP6 and its non-polar *siaM* deletion mutant strain JJK0731 in M9 minimal media supplemented with (A) glucose or *N*-acetylglucosamine (NAG) or, (B) *N*-acetylneuraminic acid (sialic acid). A. Upper two lines with symbols of solid square and circle indicate growth pattern in M9+glucose and lower two are of M9+NAG. Plots are represented on natural log scale. O.D., 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’s 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 reference (housekeeping) gene (mdh) in *V. vulnificus* strain CMCP6 in presence of glucose (G) or sialic acid (S). cDNA samples were diluted 1:25 and 1:125 and used as templates for semi quantitative reverse transcription-PCR reactions. Genomic DNA of CMCP6 and PCR reaction mixture without any DNA were used as positive (+) and negative (-) controls, respectively. Numbers are the molecular mass (bp) standard of 1 kb plus DNA ladder (Invitrogen).
**Vibrio vulnificus**
Chromosome II

**V. cholerae**
Chromosome I (VPI-2)

**V. mimicus**
Chromosome II (VPI-2)

**V. orientalis**

**V. fischeri**
Chromosome I

**Photobacterium profundum**
Chromosome I

---

Fig. 1
Fig. 3

(A) VVA1198-VVA1206
SAT & SAC (12 kb)

(B) VVA1198-VVA1206
rpiR nanA siaMQP nanE nanK

(C) VV2_0729-VV2_0736
~18 kb

1.3 kp
2.2 kp
2.3 kp
4.6 kb

(D) vva1194-vva1212
~18 kb
~7 kb

(E) nanA-nanE
4.6 kb

(F) rpiR-nanA
nanA-siaQ
siaQ-nanE

Fig. 3
Fig. 4

(A) M9 + Glucose

(B) M9 + Sialic acid

***
Fig. 5