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Genomic and Metabolic Profiling of Nonulosonic Acids in *Vibrionaceae* Reveal Biochemical Phenotypes of Allelic Divergence in *Vibrio vulnificus*▽

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Nonulosonic acids (NulOs) encompass a large group of structurally diverse nine-carbon backbone α-keto sugars widely distributed among the three domains of life. Mammals express a specialized version of NulOs called sialic acids, which are displayed in prominent terminal positions of cell surface and secreted glycoconjugates. Within bacteria, the ability to synthesize NulOs has been demonstrated in a number of human pathogens and is phylogenetically widespread. Here we examine the distribution, diversity, evolution, and function of NulO biosynthesis pathways in members of the family *Vibrionaceae*. Among 27 species of *Vibrionaceae* examined at the genomic level, 12 species contained *nab* gene clusters. We document examples of duplication, divergence, horizontal transfer, and recombination of *nab* gene clusters in different *Vibrionaceae* lineages. Biochemical analyses, including mass spectrometry, confirmed that many species do, in fact, produce di-N-acetylated NulOs. A library of clinical and environmental isolates of *Vibrio vulnificus* served as a model for further investigation of *nab* allele genotypes and levels of NulO expression. The data show that lineage I isolates produce about 20-fold higher levels of NulOs than lineage II isolates. Moreover, *nab* gene alleles found in a subset of *V. vulnificus* clinical isolates express 40-fold higher levels of NulOs than *nab* alleles associated with environmental isolates. Taken together, the data implicate the family *Vibrionaceae* as a "hot spot" of NulO evolution and suggest that these molecules may have diverse roles in environmental persistence and/or animal virulence.

Nonulosonic acids (NulOs) are a family of negatively charged nine-carbon backbone α-keto sugars that include the neuraminic (also known as sialic), legionaminic, and pseudaminic acids (2, 35). The sialic acids are the best-understood NulOs and are found in prominent outermost positions on the surfaces of all vertebrate cells (55). In mammals, the most common NulO is sialic acid, a molecule found at particularly high levels at mucosal surfaces of mammals. Pseudaminic and legionaminic acids are not expressed in animals. In their various locations on microbial surfaces, different NulO structures have been implicated in a variety of host-microbe interactions. NulOs of the sialic, legionaminic, and pseudaminic acid types are involved in bacterial behaviors like biofilm formation, autoagglutination, and motility (1, 15, 18, 45, 52), as well as direct protein-carbohydrate interactions between hosts and pathogens (8, 9, 26, 30, 52). In particular, sialic acid-containing bacterial glycans participate in strategies of immune suppression and subversion, likely contributing to clinical conditions ranging from urogenital, airway, and ear infections to systemic bacteremia, meningitis, and the induction of autoimmunity (1, 24, 32, 56, 57, 63, 64).

Sialic acids were once thought to be unique to the deuterostome lineage of "higher" animals and absent from most protostomes, fungi, plants, and protists (61). In fact, the biosynthetic pathways for sialic acids appear to be quite ancient, likely predating the divergence of the three domains of life (35). Recent studies show that NulO biosynthetic (*nab*) gene clusters are found in a surprising variety of bacterial strains and species, including a large number of gammaproteobacteria, including the family *Vibrionaceae* (35). Different proteobacteria have been shown to express NulOs as modifications of polymerized cell surface molecules such as capsular polysaccharides (16, 34), lipopolysaccharides (LPSs) (37, 48, 51), and flagella (40–43, 50). Among the members of the family *Vibrionaceae*, strains of *Vibrio parahaemolyticus*, *V. vulnificus*, *Aliivibrio salmonicida*, and *Photobacterium profundum* have also been shown to express NulOs (7, 13, 35, 58). However, little is known about the larger distribution patterns, the natural history of NulOs in *Vibrionaceae*, or the biology of these molecules in aquatic or host-associated niche environments.

As a group, the members of the family *Vibrionaceae* engage in a full spectrum of lifestyles, from free-living states to colonization or infection of both aquatic and terrestrial hosts (3, 17). *V. vulnificus* is an excellent example of the range of environmental and host niches that can be occupied by different members of the same *Vibrio* species. *V. vulnificus* is an obligate halophile found in estuarine and marine coastal environments worldwide (28, 29, 46, 53). It is found in association with zooplankton, crabs, and various filter feeders, such as oysters

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TABLE 1. Species and strains examined in this study

Species	Isolate name
Listonella anguillarum	B559
Listonella anguillarum	B561
Listonella anguillarum	B563
Vibrio angustum	B70
Vibrio fischeri	ATCC 33983
Vibrio fischeri	394
Vibrio fischeri	63
Vibrio fischeri	ES114
Vibrio harveyi	ATCC 33843
Vibrio harveyi	L222
Vibrio logei	B583
Vibrio ordalii	B572
Vibrio orientalis	B717
Vibrio pelagius	B98
Vibrio splendidus	B12
Vibrio parahaemolyticus	428/01
Vibrio parahaemolyticus	30824
Vibrio parahaemolyticus	9808/1
Vibrio parahaemolyticus	UCMA178
Vibrio parahaemolyticus	090-96
Vibrio parahaemolyticus	906-97
Vibrio parahaemolyticus	190-2004
Vibrio parahaemolyticus	155-05
Vibrio parahaemolyticus	357-99
Vibrio parahaemolyticus	518-01
Vibrio parahaemolyticus	VP81
Vibrio parahaemolyticus	Vphy191
Vibrio parahaemolyticus	V0441
Vibrio parahaemolyticus	V0586

and mussels (12, 21, 22, 44). *V. vulnificus* is also a highly invasive pathogen of both fish and humans, and in humans, infection is characterized by primary septicemia and wound infections with mortality rates of greater than 50% among susceptible individuals (27, 38, 47). Multilocus sequence typing analysis of six housekeeping genes and genotyping data have previously divided *V. vulnificus* isolates into at least three distinct clusters or lineages; lineage I is composed exclusively of biotype 1 isolates recovered mainly from clinical sources, lineage II contains biotype 1 and all biotype 2 isolates recovered from environmental sources, including diseased fish, and the third lineage is composed of biotype 3 (4–6, 11, 59, 60). Biotype 3 isolates, which are recovered from one geographic region and associated with one fish species, were shown to be genetically identical and distinct from lineage I and II isolates (4, 5).

We hypothesize that the family *Vibrionaceae* may be a particularly active lineage of NulO evolution. Here we combine genomic and biochemical approaches to more systematically investigate and document the distribution, phylogeny, and functional activity of homologous NulO biosynthetic (NAB) pathways in members of the family *Vibrionaceae*.

(A portion of this work was presented by S.A. at the 2009 INTEL International Science and Engineering Fair.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Tables 1 and 2. Unless otherwise noted, 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). The 67 *V. vulnificus* isolates examined in this study were temporally (1980 to 2005) and geographically (Asia, Europe, and North America) widespread, encompassing all 3 biotype groups as previously reported (11). Of the 67 isolates examined, 27 were recovered from clinical sources (wound infections and blood) and 40 were from environmental sources (clams, oysters, mussels, fish, seawater, and sea sediment). All *V. vulnificus* strains were grown in LB supplemented with 2% NaCl and stored at −80°C in LB broth with 20% (vol/vol) glycerol.

Molecular analysis. Chromosomal DNA was extracted from each of the *V. vulnificus* isolates using the Genome DNA isolation kit from Bio 101 as previously described (11) (MP Biomedicals, Solon, OH). The PCR primers used to amplify the *nab1* and *nab2* genes from *V. vulnificus* YJ016 and CMCP6 were designed based on unique sequences of each strain and purchased from Integrated DNA Technologies (Coralville, IA) (Table 3). PCRs were performed in a 25-μl reaction mixture using the following program: an initial denaturation step of 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 s, 30 s of primer annealing at 57 to 61°C, and 60 s of primer extension at 72°C. PCR products were visualized on agarose gels.

Bioinformatic and phylogenetic analysis of *nab* genes. We performed BLAST searches (blastp) against the sequenced genome database using as seeds the sequences of proteins encoded by *nab1*, *nab2*, *nab3*, and *nab4* from YJ016 and CMCP6, respectively. Phylogenetic analysis was performed on Nab1 and Nab2 from 21 strains encompassing 12 species. The *nab1* gene alleles from 2 of the 3 *nab* clusters in *A. salmonicida* are pseudogenes and were not included in the analysis of Nab1 proteins. Complete protein sequences were aligned using ClustalW 2.0 or MUSCLE V3.8, and the alignment was manually checked for invariant and conserved regions using GENEDOC (version 2.7; National Resource for Biomedical Supercomputing (Pittsburgh, PA) (14, 33). We used neighbor joining (NJ) as implemented in MEGA4 (19, 20, 54). The bootstrap values for NJ trees were obtained after 1,000 generations, and MEGA4 tree viewer was used to visualize the trees and calculate confidence values (25, 54). 16S rRNA sequences were also analyzed as described above for overlay of NAB pathway presence or absence.

Nucleotide sequence accession numbers. The 16S rRNA gene sequence accession numbers used to construct *Vibrio* phylogeny were as follows: *Vibrio cholerae* CECT 514, X76337; *Vibrio furnissii* ATCC 35016, X76336; *Vibrio fluvialis* NCTC 11327, X76335; *Vibrio brasiliensis* LMG 20546, AJ316172; *Vibrio mimicus* ATCC 33653T, X74713; *Vibrio metschnikovii* CIP69.14T, X74711; *V. vulnificus* ATCC 27562, X76333; *Vibrio alginolyticus* ATCC 17749, X56576; *Vibrio splendidus* LMG 4042, AJ515230; *Vibrio parahaemolyticus* ATCC 17802, AF388386; *Vibrio harveyi* NCIMB1280T, AY750575; *Vibrio tubiashii* ATCC 19109, X74725; *Vibrio orientalis* ATCC 33934T, X74719; *Vibrio sinaloensis* CAIM 797, DQ451211; *Vibrio rotiferatus* LMG 21460, AJ316187; *Vibrio navarrensis*, X74715; *Vibrio* sp. AND4, AF025960; *Vibrio* sp. EX25, CP001805; *Vibrio coralliilyticus* LMG 20984, AJ440005; *Vibrio shiloni* AK1, AF007115.1; *Listonella anguillarum*, AM235737; *Photobacterium damselae* ATCC 33539, AB032015; *Photobacterium angustum* ATCC 25915, D25307; *Photobacterium profundum* DSJ4, D21226; *Grimontia hollisae* LMG 17719, AJ514909; *Aliivibrio fischeri* ATCC 774T, X74702; *Aliivibrio salmonicida* NCMB 2262, X70643; *Vibrio ordalii* ATCC 33509T, X74718; *Shewanella benthica* ATCC 43992, X82131. PCR primers for *V. vulnificus* YJ016 and CMCP6 *nab1* and *nab2* were designed using accession numbers VV0316 (NP_933109.1), VV0312 (NP_933105.1), VV10803 (NP_759780.1), and VV10808 (NP_759785.1) respectively. The accession numbers for the other *Vibrio nab* genes used, including those used for BLAST analysis and tree construction, are as follows. The *nab1* accession numbers for *V. vulnificus* (2 strains), *Vibrio* sp. EX25, *V. parahaemolyticus*, *V. salmonicida*, *V. fischeri*, *Photobacterium profundum* (2 strains), *V. harveyi*, *V. splendidus*, *V. coralliilyticus*, *V. shilonii*, *V. mimicus*, and *Grimontia hollisae* are NP_759780.1, NP_933109.1, ZP_04922879.1, NP_796582.1, YP_002264336.1, YP_203530.1, YP_002154944.1, ZP_01218688.1, YP_130889.1, ZP_00989910.1, YP_001443906.1, ZP_01867489.1, ZP_06040339.1, ZP_06054134.1, and ZP_06053975.1. The *nab2* accession numbers for *V. vulnificus* (2 strains), *Vibrio* sp. EX25, *V. parahaemolyticus*, *V. salmonicida*, *V. fischeri*, *P. profundum* (2 strains), *V. harveyi*, *V. splendidus*, *V. coralliilyticus*, *V. shilonii*, *V. mimicus*, and *G. hollisae* are NP_759785.1, NP_933105.1, ZP_04922882.1, NP_796579.1, YP_002261714.1, YP_002261791.1, YP_002264335.1, YP_203526.1, YP_002154946.1, ZP_01218684.1, YP_130887.1, YP_001443899.1, ZP_00989905.1, ZP_05888524.1, ZP_01867488.1, ZP_06040343.1, ZP_06054132.1, and ZP_06053977.1.

Release of NulOs by mild acid hydrolysis. NulO sugars were released from extensively washed culture pellets by mild acid hydrolysis using 2 N acetic acid for 3 h at 80°C as previously described (36). Supernatants were filtered over Centricon centrifugal filtration cassettes with a molecular weight cutoff of 10,000 (Millipore, Kankakee, IL). The low-molecular-weight fraction was then lyophilized and stored at −20°C for use in thiobarbituric acid (TBA), 1,2-diamino-4,5-methylene dioxybenzene (DMB) high-performance liquid chromatography (HPLC), or mass spectrometric analyses as described below.

TBA assays. The TBA assay is relatively simple, fast, and inexpensive and has been used extensively for studies of NulOs such as *N*-acetylneuraminic acid.

TABLE 2. *V. vulnificus* strains used in this study and their *nab* alleles

Strain name	*nab1* allele	*nab2* allele	Place of isolation	Source	Yr of isolation
YJ002	YJ016-like	YJ016-like	Taiwan	Clinical	1993
JJ068			Taiwan	Clinical	1993
JJ072			Taiwan	Clinical	1993
JJ067		YJ016-like	Taiwan	Clinical	1993
YJ016	YJ016-like	YJ016-like	Taiwan	Clinical	1993
L-180			Japan	Clinical	1980
N-87	CMCP6-like	CMCP6-like	Japan	Clinical	1987
KH-03	YJ016-like	YJ016-like	Japan	Clinical	2003
SPRC 10143	YJ016-like	YJ016-like	NK[a]	Clinical	NK
CMCP6	CMCP6-like	CMCP6-like	Korea	Clinical	NK
CDC 9038-96	CMCP6-like	CMCP6-like	Texas	Clinical	1996
CDC 9062-96	CMCP6-like	YJ016-like	Louisiana	Clinical	1996
CDC 9005-97		YJ016-like	Louisiana	Clinical	1997
CDC 9030-95	YJ016-like	YJ016-like	Florida	Clinical	1995
313-98			Israel	Clinical	1998
11028			Israel	Clinical	NK
CIP 81.90	YJ016-like	YJ016-like	France	Clinical	1980
LSU 1866		YJ016-like	NK	Clinical	NK
M06	CMCP6-like	CMCP6-like	United States	Clinical	NK
M06-24/O	CMCP6-like	CMCP6-like	California	Clinical	NK
6353/O	YJ016-like		Maryland	Clinical	NK
85A667/O		YJ016-like	California	Clinical	NK
K2637	CMCP6-like		Louisiana	Clinical	2005
K2667	CMCP6-like	CMCP6-like	Louisiana	Clinical	2005
K2719	YJ016-like	YJ016-like	Louisiana	Clinical	2005
C-7184			United States	Clinical	NK
IFVv8			Florida	Clinical	NK
CG27	CMCP6-like	CMCP6-like	Taiwan	Oyster	1993
98-640 DP-B9	YJ016-like	YJ016-like	Louisiana	Oyster	1998
99-609 DP-A4	YJ016-like	YJ016-like	Oregon	Oyster	1999
99-779 DP-D2		YJ016-like	Louisiana	Oyster	1999
99-509 DP-A6	YJ016-like	YJ016-like	Texas	Oyster	1999
99-540 DP-B6	YJ016-like	YJ016-like	Texas	Oyster	1998
300-1C1	YJ016-like	YJ016-like	NK	Oyster	NK
JY1701		YJ016-like	Louisiana	Oyster	NK
Env1	YJ016-like	YJ016-like	Louisiana	Oyster	NK
SS108A-3A	YJ016-like	YJ016-like	NK	Oyster	NK
b60	CMCP6-like	CMCP6-like	India	Oyster	NK
b122	CMCP6-like	CMCP6-like	India	Oyster	NK
IFVv10			France	Mussel	NK
IFVv11			France	Mussel	NK
72M4	CMCP6-like	CMCP6-like	India	Clam	NK
79M4	YJ016-like	YJ016-like	India	Clam	NK
76M3	YJ016-like	YJ016-like	India	Fish	NK
80M4	CMCP6-like	YJ016-like	India	Fish	NK
G-83	YJ016-like	YJ016-like	Korea	Fish	NK
NCIMB 2136	YJ016-like	YJ016-like	Japan	Eel	NK
90-2-11	YJ016-like	YJ016-like	Norway	Eel	NK
ATCC 33149	YJ016-like	YJ016-like	Japan	Eel	NK
NCIMB 2137	YJ016-like	YJ016-like	Japan	Eel	NK
E86	YJ016-like	YJ016-like	Spain	Eel	1990
M79	CMCP6-like	CMCP6-like	Spain	Eel	NK
IFVv18			France	Seawater	NK
MLT362	CMCP6-like	CMCP6-like	Hawaii	Seawater	1991
MLT364	CMCP6-like	CMCP6-like	Hawaii	Seawater	1991
MLT406	YJ016-like		Florida	Seawater	1991
SPRC 10215			NK	Seawater	NK
CG62		YJ016-like	Taiwan	Seawater	1993
CG63	YJ016-like	YJ016-like	Taiwan	Seawater	1993
CG123	YJ016-like	YJ016-like	Taiwan	Seawater	1993
96-9-114s			Denmark	Sediment	NK
L-49			Japan	Environmental	1988
JY1305	YJ016-like	YJ016-like	Louisiana	Environmental	NK
UNCC 1015	CMCP6-like	CMCP6-like	North Carolina	Environmental	NK
MLT 365	CMCP6-like	CMCP6-like	Florida	Environmental	NK
UNCC 913	YJ016-like	YJ016-like	North Carolina	Environmental	NK
345/O	CMCP6-like	CMCP6-like	Louisiana	Environmental	NK

[a] NK, not known.

TABLE 3. Primers used for PCR assays in this study

Oligonucleotide name	Sequence (5′–3′)	Product size (bp)	T_m (°C)
VV0312F	CGA CGA AGC ACT GGC GTT TAA A		
VV0312R	GCT CGA GCA TCT CCC AAT ACT	986	61
VV0316F	GGC CAC CCC TTC AAT TGA G		
VV0316R	GTC GCA TAC ACA ACC GTG G	435	60
VV10808F	TAT TCG TTT AGC CAA ACA GTT GA		
VV10808R	CCA CTT CAT CCC AAC GCG TT	902	57
VV10803F	TTA TCG GCG ACA AGG TGA		
VV10803R	ATC CAT TAC ATA GGC AAA TAT G	346	60

Potential NulOs released from vibrios (see above) were treated for 30 min at 37°C with 0.1 N (final concentration) sodium hydroxide, followed by neutralization as previously described (23). The *Vibrio* strains used in these assays are listed in Table 1. Measurement of TBA-reactive species (TBARs) in this material was performed as previously described (62). Group B and A streptococci were used as positive and negative controls, respectively; the former display high levels of surface sialic acids, while the latter do not. A standard curve for TBARs was generated in each experiment using *N*-acetylneuraminic acid (Sigma). Results were normalized to the total protein contents of bacterial culture pellet lysates that were set aside prior to NulO hydrolysis. Protein content was measured using the bicinchoninic acid assay (Pierce).

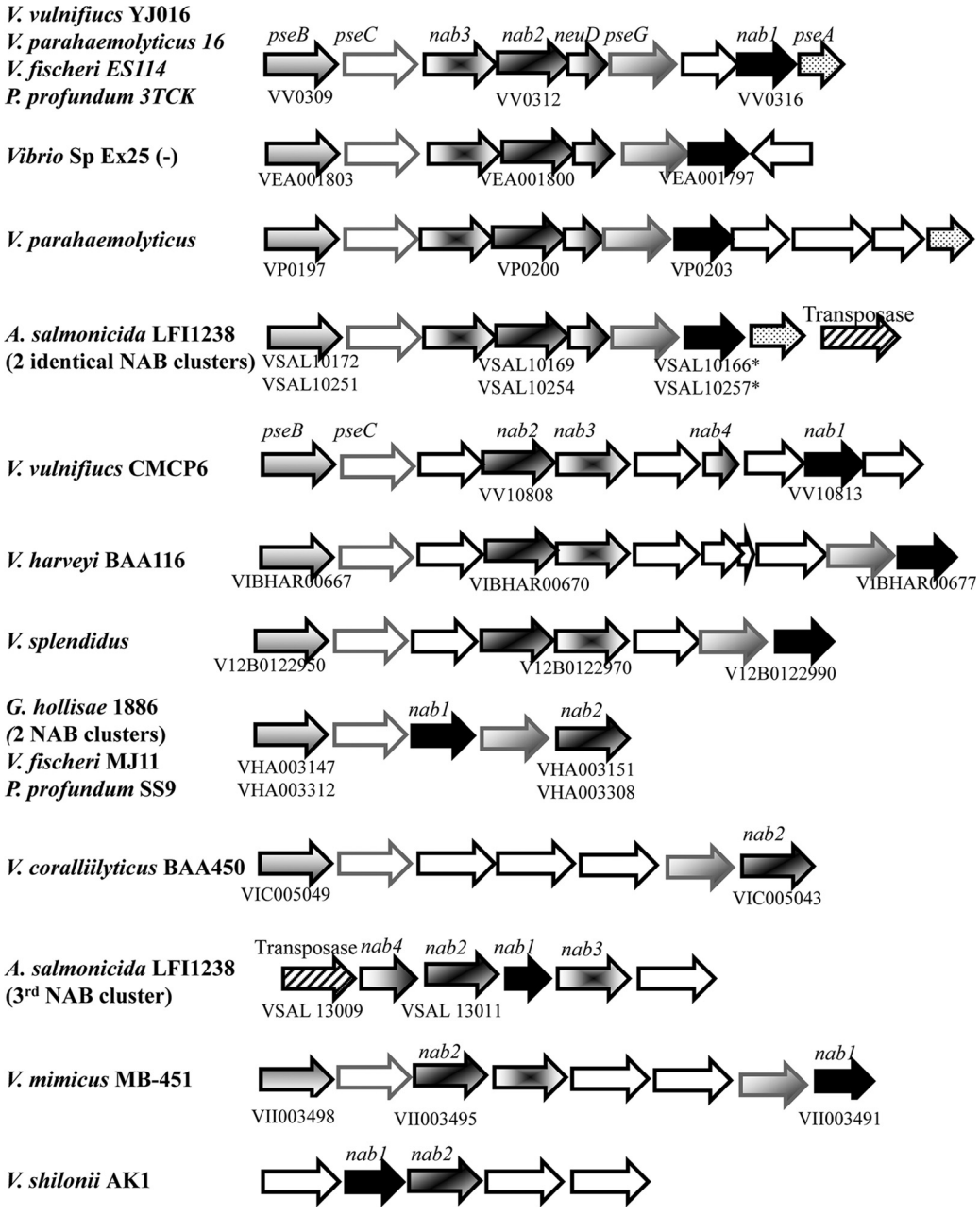


FIG. 1. Genetic structure of the *nab* gene cluster among sequenced *Vibrionaceae* species. Shown is a schematic representation of the gene arrangement of NAB clusters among sequenced isolates of members of the family *Vibrionaceae*. The annotated homologs of the ORFs are *nab1* (CMP-NeuAc synthase homolog), *nab2* (*N*-acetylneuraminic acid synthase homolog), *nab3* (UDP–*N*-acetylglucosamine 2 epimerase homolog), *neuD* (acetyltransferase homolog), *pseA* (flagellin modification protein homolog), *pseB* (polysaccharide biosynthesis homolog), *pseC* (DegT aminotransferase homolog), and *pseG* (nucleotidyl/sugar P transferase homolog). Strains with identical gene orders are shown only once with isolate names listed on the left. Accession numbers for *nab* genes are shown below schematic ORFs.

DMB derivatization and HPLC. NulOs released by mild acid hydrolysis were derivatized with DMB. Reaction mixtures consisted of 7 mM DMB, 18 mM sodium hydrosulfite, 1.4 M acetic acid, and 0.7 M 2-mercaptoethanol, and reactions were carried out for 2 h at 50°C in the dark. DMB-NulO derivatives were resolved by HPLC using a reverse-phase C_{18} column (Varian) eluted isocratically at a rate of 0.9 ml/min over 50 min using 85% Milli-Q ultrapure water, 7% methanol, and 8% acetonitrile as previously described (35, 36). Detection of fluorescently labeled NulO sugars was achieved at excitation and emission wavelengths of 373 nm and 448 nm, respectively. 3-Deoxy-D-manno-octulosonic acid (Kdo) is an eight-carbon backbone α-keto acid that forms part of the conserved core portion of LPS in virtually all Gram-negative bacteria. Kdo was released and derivatized under the same conditions as the related NulO α-keto acids and served as an internal control in these assays to express relative levels of NulOs. The Mann-Whitney nonparametric test was used for statistical evaluation of NulO/Kdo ratio differences between strains. All of the chemicals used in DMB reactions were from Sigma, St. Louis, MO.

Electrospray ionization mass spectrometry. DMB-derivatized extracts or individually isolated HPLC peaks were analyzed at the University of California San Diego Glycotechnology Core resource using a ThermoFinnigan LCQ ion trap mass spectrometer with tandem HPLC.

RESULTS

Frequency of *nab* gene clusters in *Vibrionaceae*. As of September 2010, there were 24 species and 66 strains of the family *Vibrionaceae* in the genome database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). This includes 27 strains of *V. cholerae*, four strains of the closely related species *V. mimicus*, seven *V. parahaemolyticus* genome sequences, and 21 additional species with either two genomes or a single genome represented. NAB pathways are encoded by *nab* gene clusters, which share a core portion composed of *nab1*, a homolog of CMP–*N*-acetylneuraminic acid synthetase, *nab2*, a homolog of *N*-acetylneuraminic acid synthase, and *nab3*, a homolog of UDP–*N*-acetylglucosamine 2 epimerase. Many of these sequenced *Vibrio* species contained a putative NAB pathway. BLAST analysis of *nab* genes identified 12 species and a total of 21 strains containing *nab* gene clusters, i.e., *V. vulnificus* (2 strains), *V. parahaemolyticus* (6 strains), *V. mimicus* (1 strain), *V. harveyi* (2 strains), *V. shilonii* (1 strain), *V. splendidus* (1 strain), *V. coralliilyticus* (1 strain), *V. fischeri* (2 strains), *V. salmonicida* (1 strain), *Vibrio* sp. EX25 (1 strain), *Photobacterium profundum* (2 strains), and *Grimontia hollisae* (1 strain) (Fig. 1). However, about 50% (13/27) of the fully sequenced *Vibrionaceae* species isolates did not contain homologous pathways for NulO biosynthesis (Fig. 2).

Evolution of NulO biosynthesis in *Vibrionaceae*. Analysis of the distribution and arrangement of *nab* gene clusters among *Vibrionaceae* lineages reveals a complex evolutionary history. Within many lineages, there is a conspicuous presence or absence of homologous NAB pathways, with some notable exceptions. For example, among the seven *V. parahaemolyticus* strains examined, only AN-5034 lacked the *nab* cluster and the *nab* genes from 5/6 other strains shared 100% amino acid sequence identity. *V. parahaemolyticus* strain 16 shared only 90% amino acid identity with the *nab* genes from the other strains. Among the 27 *V. cholerae* genomes, none contained *nab* genes. Yet, within sequenced isolates of *V. mimicus*, a species closely related to *V. cholerae*, 1 of the 4 sequenced strains (MB451) contained a *nab* region with a divergent gene order compared to that of related species (Fig. 1).

For other vibrios, phylogenetic analysis of Nab amino acid sequences revealed that members of the same species sometimes contain NAB pathways that are highly divergent from each other. One of the clearest examples of phylogenetic divergence involved the two sequenced strains of *V. vulnificus* (YJ016 and CMCP6), both clinical biotype 1 strains from Asia (10). The *nab* gene cluster within *V. vulnificus* YJ016 encompasses open reading frames (ORFs) VV0311 to VV0316 on chromosome 1. In strain CMCP6, the NAB cluster encompasses ORFs VV10803 to VV10808 and is also found on chromosome 1 (Fig. 1). Both Nab1 and Nab2 from CMCP6 and YJ106 are found in two divergent lineages (a and b in Fig. 3A and B). Nab2 from YJ016 is closely related to Nab2 from *V. fischeri* ES114 and *P. profundum* 3TCK, whereas Nab2 from CMCP6 is closely related to Nab1 from *V. splendidus* and *V. harveyi* (a and b in Fig. 3B).

Representative members of other species (*A. salmonicida*

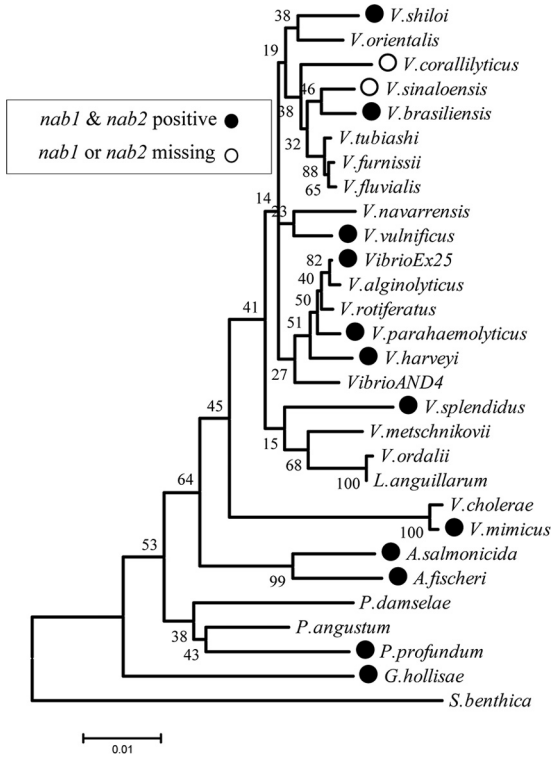


FIG. 2. Distribution of *nab* genes among sequenced members of the family *Vibrionaceae*. A phylogenetic tree of completely sequenced members of the family *Vibrionaceae* based on 16S rRNA sequences was constructed. Evolutionary history was inferred by the NJ method (49). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are expressed as the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 1,191 positions in the final data set. Phylogenetic analyses were conducted in MEGA4 (54). *Shewanella benthica* 16S rRNA served as the outgroup. Strains that contain *nab* genes are indicated by circles on the right. Accession numbers for 16S sequences are provided in Materials and Methods.

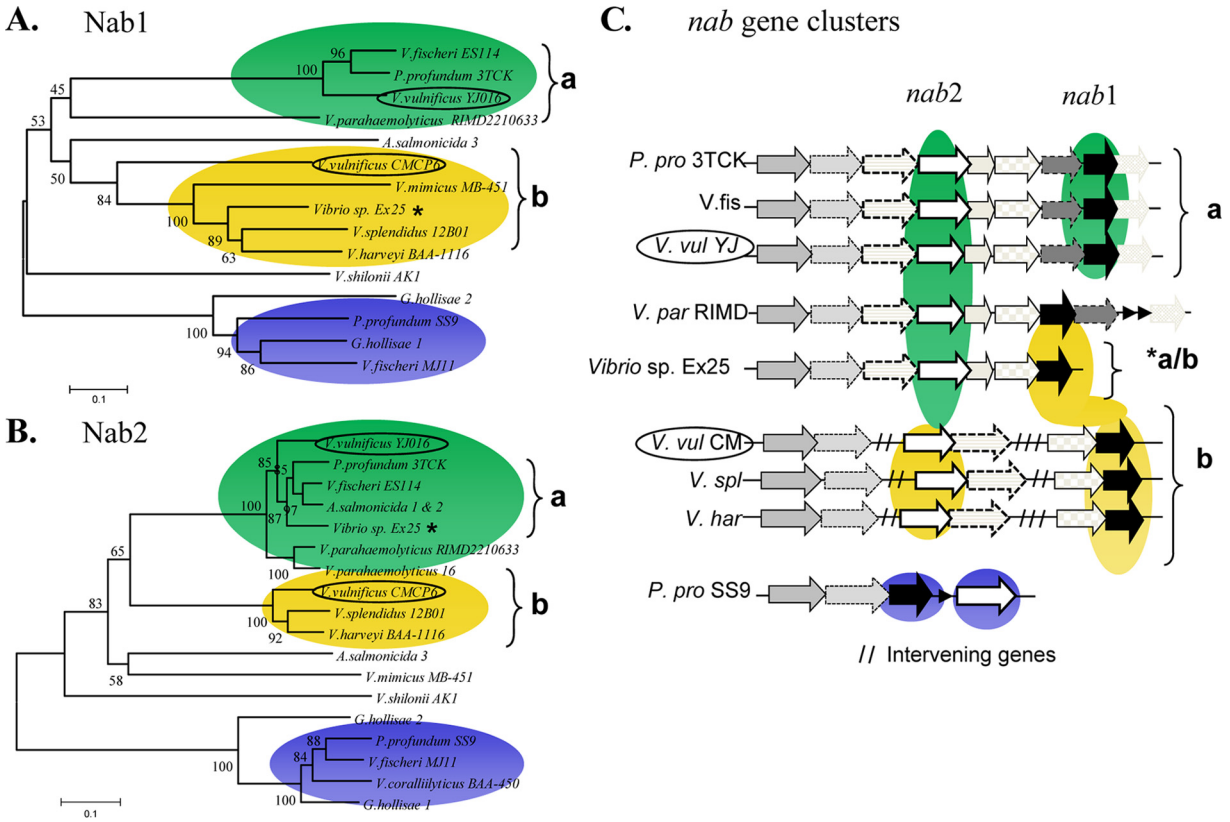


FIG. 3. Correlation of phylogenetic branching patterns and analysis of Nab1 and Nab2 among *Vibrionaceae* species. Evolutionary relationships of Nab1 (A) and Nab2 (B) amino acid sequences from 12 species of the family *Vibrionaceae* was inferred by the NJ method (49). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The phylogenetic trees are drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer each phylogenetic tree. Evolutionary distances were computed using the Poisson correction method and are expressed as the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 432 positions in the final data set. Phylogenetic analyses were conducted in MEGA4 (54). The lowercase letters a and b refer to similar phylogenetic clades and gene cluster arrangements shown in green and yellow, respectively. Asterisks highlight *Vibrio* sp. EX25, which exhibits evidence of recombination between the a and b gene cluster alleles. See Fig. 1 for a complete description of gene identifiers and accession numbers.

and *G. hollisae*) contain multiple paralogous *nab* gene clusters within the same genome. In *A. salmonicida*, ORFs VSAL_I0164 to VSAL_I0172 are duplicated at positions VSAL_I0250 to VSAL_I0259, but on the negative strand, and the *nab1* gene is a pseudogene. A third copy of the *nab* cluster is found in this species at ORFs VSAL_I3009 to VSAL_I3013, but the region shows low sequence similarity to the other two clusters and has a unique gene arrangement (Fig. 1). Flanking all three *nab* regions in *A. salmonicida* is a transposase. In the *G. hollisae* genome, two copies of the *nab* region are present but the regions show less than 65% amino acid identity, ORFs VHA_003148 to VHA_003151 and VHA_003308 to VHA_003011; however, the gene order is identical. Taken together, these observations suggest that *nab* gene clusters in *Vibrionaceae* are prone to duplication, divergence, and horizontal transfer.

Gene cluster organization matches phylogenetic signatures of Nab1 and Nab2 in *Vibrionaceae*. Initial observations of the *nab* gene clusters in sequenced strains of *Vibrionaceae* and other gammaproteobacteria suggested that there are at least three divergent *nab* gene alleles found within different gene cluster arrangements (Fig. 1). To examine this more carefully, selected *nab* gene clusters were aligned (Fig. 3C) and compared to the phylogenetic branching patterns of the Nab1 (Fig. 3A) and Nab2 (Fig. 3B) amino acid sequences. There was a strong correlation between the *nab* gene cluster arrangement and the phylogenetic lineages of the Nab1 and Nab2 amino acid sequences (Fig. 3, lineages a and b). These data also revealed examples of apparent recombination between the different *nab* alleles. For example, while most of the members of the family *Vibrionaceae* contained *nab1* and *nab2* alleles that both cluster in phylogenetic clade a or b, the *Vibrio* sp. EX25 strain contained *nab1* and *nab2* alleles that clustered in different phylogenetic clades (b and a, respectively) (Fig. 3).

Biochemical analyses of *Vibrionaceae* isolates confirm that many species express NulOs. To determine whether NAB pathways are functional in *Vibrionaceae*, analytical approaches were applied to investigate the potential production of NulOs in different lineages. Initially, *Vibrionaceae* isolates (28 strains representing 14 species) were subjected to the classic TBA assay, a method originally used to evaluate the distribution and levels of sialic acids expressed by various animal lineages (61). Indeed, many of the vibrios expressed TBARs when grown

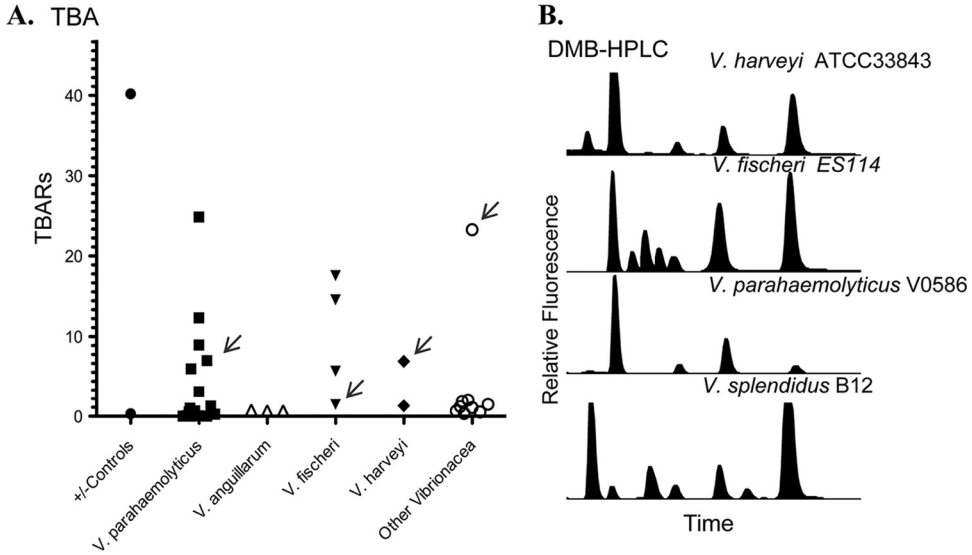


FIG. 4. Analysis of putative NulOs in *Vibrionaceae*. (A) A TBA assay of *Vibrio* strains listed in Table 1 was carried out as described in Materials and Methods. Results were normalized to total protein content and expressed as TBARs. Controls gave the expected results and included *Streptococcus agalactiae* (group B), which is known to express sialic acids, and *Streptococcus pyogenes* (group A), which does not express sialic acids (+ and −, respectively). (B) Putative NulOs released from selected strains (indicated by arrows in panel A) were fluorescently derivatized with DMB as described in Materials and Methods. DMB-derivatized α-keto acids were then resolved by HPLC with reverse-phase separation.

under standard culture conditions (Fig. 4A). However, as suggested by genomic observations of *nab* gene clusters in sequenced vibrios, many of the *Vibrionaceae* isolates did not show TBARs at levels above those observed in negative controls. The method of TBA reactivity has some limitations, including the potential for interfering molecular species that may confound interpretation of the results (62). To further characterize potential NulO expression, selected isolates were analyzed using a method of derivatization that relies specifically on the α-keto acid shared by all types of NulOs. Briefly, mild acid hydrolysates of selected *Vibrio* isolates were subjected to derivatization (fluorescence tagging) of NulOs with DMB. Molecular species of DMB-NulOs were then resolved by HPLC. Chromatograms revealed multiple peaks of DMB-derivatized molecules in several *Vibrio* isolates (Fig. 4B).

Further verification that HPLC peaks of DMB-derivatized molecules correspond to masses characteristic of NulOs was achieved by mass spectrometry (Fig. 5). For example, experiments with *V. fischeri* ES114 showed several retention times of DMB-derivatized molecules that correspond to masses of di-N-acetylated NulOs (*m*/*z* 450 to 451) (35), while *V. parahaemolyticus* V0586 and *V. vulnificus* N-87 produced a single dominant NulO species of this mass (note that 473 and 433 correspond to the sodium adduct and dehydrated forms of the same molecule). Due to the multiple epimers and modifications of NulOs that can occur naturally (31), identical masses found within many of the HPLC peaks could not be assigned unambiguously to specific chemical structures. However, it is clear from these studies that a variety of di-N-acetylated NulOs are produced by multiple strains of *V. parahaemolyticus*, *V. fischeri*, and *V. vulnificus*. Taken together, these experiments conclusively show that many members of the family *Vibrionaceae* contain functional NAB pathways that are capable of producing a variety of NulOs.

Distribution of *nab* alleles among diverse *V. vulnificus* isolates. To more clearly define potential associations between the different *nab* alleles, functional output of NulOs, and specific bacterial phylogenetic lineages, a more directed approach within a single species was utilized. *V. vulnificus* was chosen for these studies due to the phylogenetically divergent *nab* gene clusters found in the two available reference genome strains (YJ016 and CMCP6) (Fig. 3 and 6). Allele typing of *nab1* and *nab2* was performed on a collection of 67 *V. vulnificus* strains whose phylogenetic relationships are known, using a set of primer pairs for *nab1* and *nab2* alleles designed using genomic sequences from reference strains YJ016 and CMCP6 (alleles here referred to as YJ-like and CM-like [Table 2]). Four PCR assays were performed (Table 3) using genomic DNA isolated from the 67 *V. vulnificus* strains (Table 2), of which 40 were from environmental sources and 27 were from clinical sources. We then mapped the presence of YJ-like or CM-like *nab1* and/or *nab2* alleles onto the *V. vulnificus* phylogenetic tree (11) (Fig. 6).

PCR assays were negative for 13 of the 67 isolates examined, including biotype 3 isolates, suggesting that these strains may either lack the genes or contain unique untypeable *nab* genes (Fig. 6). YJ-like alleles of *nab1* and *nab2* were identified in 26 strains, while 16 strains contained CM-like alleles of both genes. Among the lineage I isolates, which are mostly from clinical sources, both YJ-like and CM-like alleles of *nab1* and *nab2* were present. However, among the lineage II isolates, which are predominantly environmental and fish isolates, the YJ-like allele was by far the most common (Fig. 6). Interestingly, two *V. vulnificus* isolates contained a CM-like *nab1* allele with a YJ-like *nab2* allele (Fig. 6). Taken together with data presented in Fig. 3, these observations further support the conclusion that members of the family *Vibrionaceae* sometimes

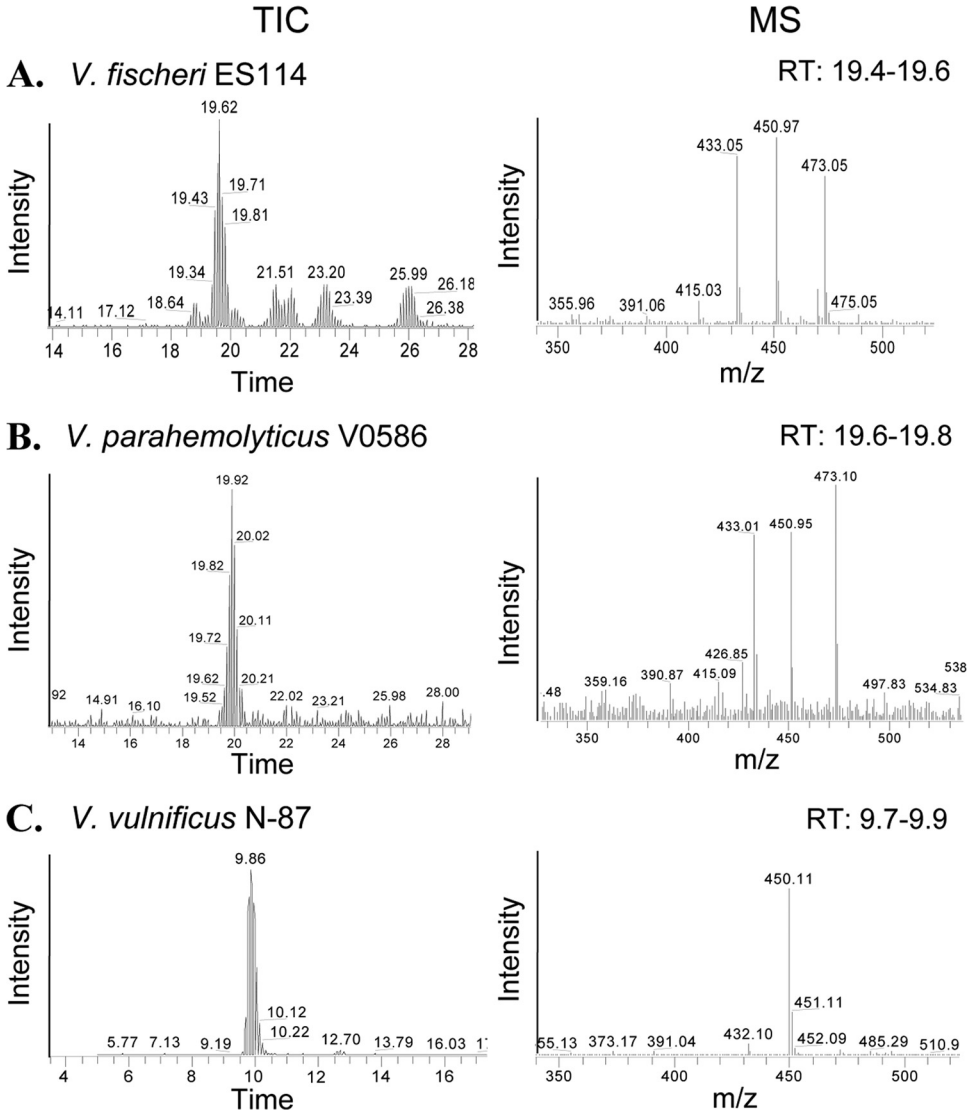
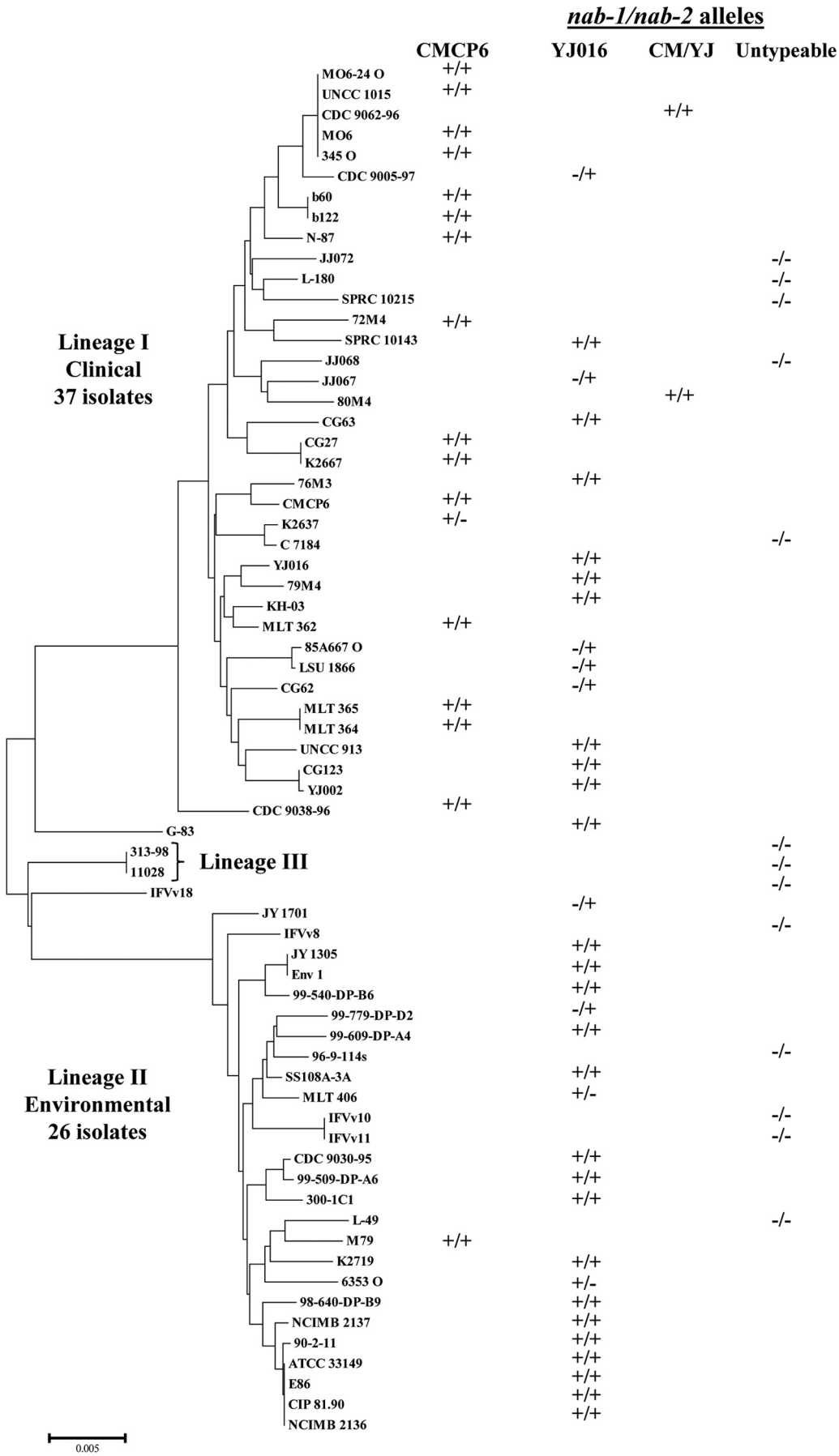


FIG. 5. Mass spectrometry reveals masses of DMB-derivatized di-N-acetylated NulOs in *Vibrionaceae*. *V. fischeri* (ES114*) (A), *V. parahaemolyticus* (518-01 and V0586*) (B), and *V. vulnificus* (CDC903896, N-87*, MLT362, MO6-24/O, and SS108A3A) (C) were subjected to electrospray ionization mass spectrometry performed in tandem with HPLC separation of DMB-NulOs. Asterisks indicate data representative of the strains tested. The total ion currents (TIC) of selected *m/z* 450 to 451 during HPLC elution are shown on the left, while mass spectra are shown on the right along with the retention times (RT).

engage in horizontal exchange and recombination of different *nab* gene cluster alleles.

NulOs are expressed at higher levels in lineage I clinical isolates with CMCP6-like alleles. We hypothesized that allelic differences in *nab* genes may predict functional differences in the level of NulO production by isolates of *V. vulnificus*. To test this hypothesis, we subjected isolates of *V. vulnificus* representing all three lineages and two *nab* allele types to DMB HPLC analysis. The structurally related 8-carbon α-keto acid known as Kdo served as a convenient internal standard for normalization of NulO expression, since it is present as part of the conserved core structure of LPS in Gram-negative bacteria and also reacts with DMB. Relative NulO production was determined for the different isolates by comparing HPLC peak areas at characteristic retention times of NulO and Kdo in *V. vulnificus* as described in Materials and Methods (Fig. 7A). Relative NulO levels were compared among lineage I and lineage II isolates (Fig. 7B) and among isolates having YJ-like and CM-like *nab1* and *nab2* alleles (Fig. 7C). These data show that NulO expression levels are much higher in isolates that contain CM-like *nab* alleles (Fig. 7C) and that these strains tend to belong to *V. vulnificus* phylogenetic lineage I (mostly clinical isolates) (Fig. 7B). Alternatively, isolates that contained YJ-like *nab* alleles expressed much lower levels of NulOs. Nearly all of the lineage II (mostly environmental) isolates contained YJ-like *nab* alleles and had low levels of NulOs (Fig. 7C). Interestingly, the data also show that most of the isolates that were "untypeable" by these methods did, in fact, express NulOs at levels that were significantly higher than those of strains with YJ-like *nab* genes but significantly lower than



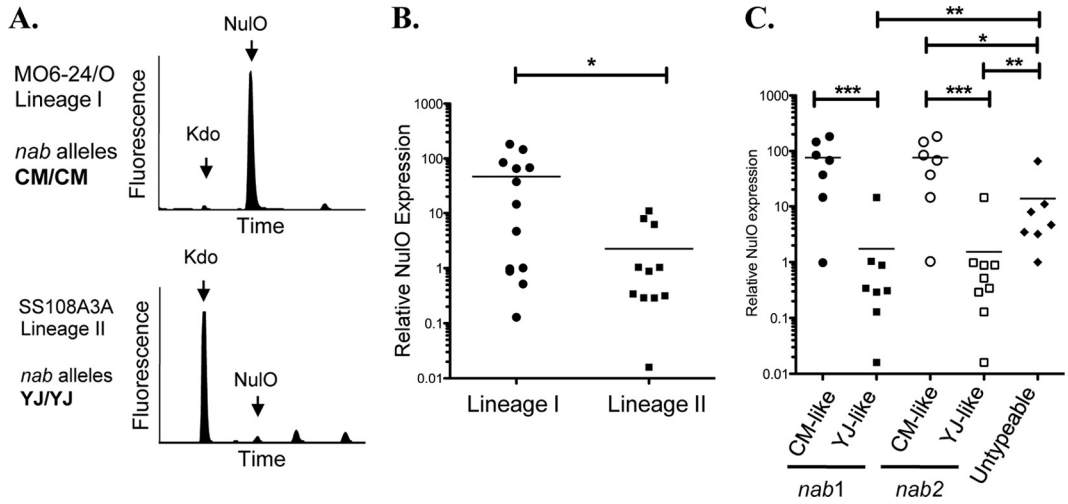


FIG. 7. CMCP6-like *nab* alleles are associated with high levels of NulO expression in mostly clinical strains. DMB HPLC analysis of diverse *V. vulnificus* isolates was carried out, and NulO expression levels were normalized to an internal control monosaccharide (Kdo) that is a part of the conserved core portion of LPS. (A) Raw HPLC data from strains representing the major *V. vulnificus* lineages (I and II) and common *nab* allele types (CMCP6 and YJ016). (B) Relative NulO expression levels in lineage I and II strains. (C) *V. vulnificus* isolates with CM-like alleles of *nab* genes have significantly higher levels of NulOs than isolates with YJ-like alleles. Most of the "untypeable" isolates do, in fact, express detectable NulOs but at levels that are intermediate compared to those of isolates with YJ-like or CM-like alleles. (B and C) The Mann-Whitney test was used for statistical evaluation (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$). Note the log scales.

those of strains with CM-like *nab* genes. Taken together, these data strongly suggest that there are at least 3 alleles of *nab* genes that correspond to low (YJ-like), intermediate (untypeable), and high (CM-like) levels of NulO expression.

DISCUSSION

These studies illustrate processes of evolution within the family *Vibrionaceae* by analysis of genetic and phenotypic patterns related to the nine-carbon backbone α-keto acid sugars (NulOs). We show that biosynthetic pathways for NulOs are widespread in members of the family *Vibrionaceae* but by no means universal (Fig. 1 and 2). The distribution and phylogeny of *nab* pathways in vibrios suggest that some lineages or strains have lost, while others have apparently duplicated, these gene clusters (Fig. 1 and 2 and Results). Multiple biochemical approaches confirm that NAB pathways are indeed active in a variety of *Vibrio* isolates (Fig. 4 and 5) and can participate in the biosynthesis of multiple structural variations of NulOs within a given strain (Fig. 5A). Genomic comparisons revealed multiple allele types of NAB pathways which are reflected by similar patterns of gene arrangement and phylogeny (Fig. 3) and correspond to functional differences in NulO expression levels (Fig. 7). In particular, clinical isolates of the CMCP6-like allele expressed, on average, nearly 100-fold higher levels of NulOs than environmental isolates with the YJ016 allele. Moreover, the data show several examples of apparent recombination between *nab* gene cluster alleles (Fig. 3 and 6). Taken together, these studies indicate a relatively plastic state of NAB pathway evolution in *Vibrionaceae*. These observations highlight the family *Vibrionaceae* and the species *V. vulnificus* as interesting models for investigations of the biological functions of NulOs.

During the completion of this study, the genome sequence of a third *V. vulnificus* strain, MO6-24/O, a clinical isolate from the United States, became available. We found that strain MO6-24/O contains a NAB cluster identical to CMCP6 and produces high levels of NulOs, confirming our genomic predictions (Fig. 6 and 7). Lastly, our data also strongly suggest the presence of a third NAB cluster type (and possibly more cluster types) that is present among natural *V. vulnificus* isolates. We identified a total of 13 strains that were untypeable by our PCR method; however, our analysis of NulO levels demonstrates that these strains produce intermediate levels of NulO, indicating the presence of *nab* genes unrelated to YJ016 or CMCP6. Given the limited number of *V. vulnificus* isolates that we examined, it is tempting to speculate that there may be even more heterogeneity in *nab* gene content within this species and the challenge going forward will be to determine the functional role of this diversity in survival and fitness.

NulOs are generally expressed on the surfaces of cells—both in "higher" animals and on bacteria. *Vibrionaceae* species likely express NulOs as part of one or more surface structures such as capsular polysaccharides, LPSs, or flagella (16, 34, 37, 40–43,

FIG. 6. Distribution of *nab1* and *nab2* allele types among *V. vulnificus*. PCR diagnosis of *nab* alleles was carried out using genomic DNA template from 67 strains of *V. vulnificus* and primers specific to the *nab1* and *nab2* alleles from reference strains YJ016 and CMCP6. If only one gene allele was positive, a minus sign was used to indicate a negative PCR result for the other allele. Strains that were negative in all PCR assays are indicated as "untypeable" here (−/−), since biochemical investigations revealed that they do, in fact, express NulOs (see Fig. 7). The phylogenetic tree of *V. vulnificus* is based on a phylogenetic analysis of six housekeeping genes as previously described (11).

48, 50, 51). For example, strains of *Campylobacter jejuni* can simultaneously express pseudaminic and legionaminic acids as flagellar modifications and sialic acids as LPS modifications (39, 40). It is possible that different *nab* alleles in *Vibrionaceae* encode slightly different NulO structures and/or that NulO sugars may be intended for different surface molecules (i.e., capsule, LPS, flagella). Alternatively, the different NAB alleles in *V. vulnificus* could be responsible for NulO modifications at different densities on the same molecule. *Vibrio* NulOs may be involved in motility, biofilm formation, relative phage susceptibility, or other phenotypes relevant to the marine ecosystem niche. In addition, *Vibrio* NulOs may have functional implications during opportunistic pathogenesis in aquatic or terrestrial animals.

In summary, the further study of NulO biology in *Vibrionaceae* will require careful analysis of the genetics and biochemistry of cell surface NulO modifications. Taken together with the many known roles of NulOs in host-microbe interactions, these studies provide a basis for further investigations of NulOs in bacterial behaviors and host-pathogen interactions involving the members of the family *Vibrionaceae*.

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