The neuraminidase gene is present in the non-toxigenic *Vibrio cholerae* Amazonia strain: a different allele in comparison to the pandemic strains

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The neuraminidase gene, nanH, is present in the O1, non-toxigenic Vibrio cholerae *Amazonia strain. Its location has been assigned to a 150 kb NotI DNA fragment, with the use of pulsed-field gel electrophoresis and DNA hybridization. This NotI fragment is positioned inside 630 kb SfiI and 1900 kb I-CeuI fragments of chromosome 1. Association of the pathogenicity island VPI-2, carrying nanH and other genes, with toxigenic strains has been described by other authors. The presence of nanH in a non-toxigenic strain is an exception to this rule. The Amazonia strain nanH was sequenced (Genbank accession No. AY825932) and compared to available* V. cholerae *sequences. The sequence is different from those of pandemic strains, with 72 nucleotide substitutions. This is the first description of an O1 strain with a different nanH allele. The most variable domain of the Amazonia NanH is the second lectin wing, comprising 13 out of 17 amino acid substitutions. Based on the presence of nanH in the same region of the genome, and similarity of the adjacent sequences to VPI-2 sequences, it is proposed that the pathogenicity island VPI-2 is present in this strain.*

Key words: *Vibrio cholerae* - Amazonia strain - neuraminidase - sialidase - *nanH* - pathogenicity island

Vibrio cholerae is a Gram-negative bacterium that lives in aquatic environments, often associated with plankton and other marine organisms (Colwell et al. 1977). Although more than 200 serogroups of *V. cholerae* have been identified, only a few (O1 and O139) are related to epidemic human disease (Glenn Morris et al. 1994). These pathogenic strains have acquired the capacity to survive adverse conditions in the host, and to multiply in the human small intestine, after ingestion of contaminated food and water. They are released through feces in the water, in a highly infectious stage (Merrell et al. 2002), and survive in the aquatic life cycle.

V. cholerae is the etiological agent of cholera, a severe diarrheal disease, with high morbidity and mortality, if left untreated (Sack et al. 2004). The disease is characterized by voluminous watery stools, dehydration, and hypovolemic shock. Cholera occurs in outbreaks, frequently affecting whole countries, and seven pandemics have been recorded (Barua 1992). The ongoing seventh pandemics started in 1961 in Indonesia, and reached Latin America in 1991, after a century of absence of reported cholera cases in this continent.

Although there are no known absolute markers that define epidemic strains, as a general rule they produce both the cholera toxin (CT) and toxin-regulated pilus (TCP) (Faruque et al. 1998). The bacteria adhere and colonize the epithelium of the small intestine by means of the TCP, and release CT in the vicinity of their target cells (Lee et al. 1999). Genes encoding CT reside in the lysogenic phage CTXΦ, and those encoding products for the biosynthesis of TCP constitute a pathogenicity island, VPI (Waldor & Mekalanos 1996, Karaolis et al. 1998). The main virulence-related factors of *V. cholerae* are thus encoded on mobile genetic elements, probably acquired via horizontal gene transfer (Faruque & Mekalanos 2003).

Cholera in Latin America was caused by a seventh pandemic El Tor strain (Salles et al. 1993, Tauxe et al. 1994). Probably due to increased surveillance during the first epidemic year in Brazil, other *V. cholerae* were isolated from patients with cholera symptoms. One particular new strain was described from a localized outbreak, and denominated the Amazonia strain (Coelho et al. 1995). *V. cholerae* Amazonia is an O1, non-toxigenic strain, isolated from more than 20 patients. It is strongly hemolytic, but other virulence factors such as TCP, zonula ocludens toxin, and thermo-stable toxin ST are absent (Coelho et al. 2000).

V. cholerae neuraminidase, a sialidase, is also considered a virulence factor (Staerk et al. 1974, Galen et al. 1992). Sialidases are found mainly in higher eukaryotes, and also in some microorganisms (Roggentin et al. 1993). *V. cholerae* neuraminidase (EC 3.2.1.18) releases sialic acid from higher gangliosides present on eukaryotic cells surface, exposing ganglioside GM1, which is the cholera toxin receptor (Holmgren et al. 1975). Recently, Jermyn and Boyd (2002) showed that the neuraminidase gene, *nanH*, is present inside a region fulfilling all the criteria to be considered a pathogenicity island. They named this new island as VPI-2, and showed that it is present in its original version in toxigenic strains, being absent in non-toxigenic strains, and present in modified versions in O139 epidemic strains, and also in the sister species *V. mimicus* (Jermyn & Boyd 2002, 2005).

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In this paper we report the presence of *nanH* in *V. cholerae* Amazonia, sequence the whole gene, compare it to nucleotide and amino acid sequences available for this gene, and map it to a 150 kb *Not*I fragment of chromosome 1, and the corresponding genomic fragments with the *Sfi*I and I-*Ceu*I enzymes.

MATERIALS AND METHODS

Bacterial strains and media - *V. cholerae* strains used in this study are shown in Table I. Cultures were started from frozen stocks using Luria-Bertani (LB) broth in a rotary shaker at 37°C.

Chromosomal DNA preparations and pulsed-field gel electrophoresis (PFGE) - Bacterial cells in late-logarithmic phase of growth were embedded in low-melting agarose plugs (USB), lysed and treated for DNA preparation essentially as previously described (Maslow et al. 1993). In-gel digestions were performed using standard protocols with the enzymes *Sfi*I, *Not*I and I-*Ceu*I. Pulsed-field-certified agarose (USB) was used in gels in 0,5X Tris-borate-EDTA electrophoresis buffer. PFGE was carried out at 10ºC with a Gene Navigator System (Amersham) with different pulse programs, according to the fragment sizes to be separated. Program 1: pulses from 5 s to 30 s, for 22 h, 1% gels; program 2: pulses from 15 s to 100 s, for 27 h, 1.2% gels; program 3, pulses from 80 s to 120 s, for 40 h, 1.2% gels. Low-range PFG marker (New England Biolabs) and yeast DNA-PFGE markers (Amer-sham) were used as size markers. After electrophoresis, the gels were stained with ethidium bromide, and photographed.

Southern blots - Southern blots followed a standard procedure (Sambrook et al. 1989), including a preliminary HCl 0.25 N treatment, for 15 min. Nylon membranes (Hybond-XL, Amersham) were used, and probes were radioactively labeled with ^{32}P-dCTP using a random primers kit (Invitrogen). Hybridization solutions contained 50% formamide, and hybridizations were carried out at 42°C. X-ray films (Hyperfilm, Amersham) were exposed overnight at –80ºC with an intensifier screen.

Primer design and PCR amplification conditions - The primers shown in Table II were used in PCR amplifications, and were designed with the use of the available genomic sequence of *V. cholerae* O1 El Tor strain N16961 (Heidelberg et al. 2000), and the Oligo v.4.0 software. PCRs were

TABLE I

Vibrio cholerae strains used in this study

Strain [a]	Description	Isolation
N16961	El Tor biotype	Bangladesh, India, 1975
3439	Amazonia strain	Tonantins, Brazil, Jan. 1992
3506	Amazonia strain	São Paulo de Olivença, Brazil, Jan. 1992
3509	Amazonia strain	São Paulo de Olivença, Brazil, Jan. 1992
4008	Amazonia strain	São Paulo de Olivença, Brazil, Apr. 1992
4010	Amazonia strain	São Paulo de Olivença, Brazil, Apr. 1992
4132	Amazonia strain	Santo Antonio do Içá, Brazil, May 1992

a: all the strains belong to serogroup O1. The Amazonia strains are serotype Ogawa, and were obtained from clinical sources.

TABLE II

Oligonucleotide primer pairs used in this study

Name	Sequence 5' – 3'	5'-end coordinate [a]	Source
nanH-f	TTTTTACAGCGTCTATGATG	1934298	This study
nanH-r	GGTTTCCTTGTGGGTTAGTA	1935424	
*LnanH*2814f	AGCCGCCGCCACTGTATTA	1932814	This study
*LnanH*3626r	TCCACCACTGAGCACTTTC	1933626	
*LnanH*2407f	ATGTCGCCTTTTGAGAGTC	1932407	This study
*LnanH*3766r	TATTCCGTTGCTGCTGTGC	1933766	
*nanH*1359f	GGCGAATGACGACAGAAA	1933589	This study
*nanH*2262r	CATCGGCAACTTGTATCT	1934509	
*nanH*2784f	TCTACCCAGCGATTGTGC	1935014	This study
*nanH*3473r	CGGTGACGAGAAATAAGC	1935720	
*nanH*2838f	GTGATGATGGCGGTTCAA	1935068	This study
*nanH*3953r	ATCTCTGCTGCTTCTTCC	1936200	
toxT f59	ATGATTGGGAAAAAATCTT	899896	Baptista 2000
toxT r859	AACTCCTGTCAACATAAAT	900714	

a: coordinates in the El Tor strain N1696 chromosome 1, as in TIGR-CMR (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gvc).

performed in 50 µl volumes containing 100 ng genomic DNA, 0.5 µg of each primer, 2 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (Biotools). The thermocycler program included an initial 5 min denaturation at 94°C, and 35 cycles at 94°C for 60 s, 55°C for 90 s and 72°C for 60 s.

DNA sequence analysis and comparisons - PCR products used for sequencing were purified using the GFX PCR and gel band purification kit (Amersham). Sequencing reactions were done with a DYEnamic ET dye terminator kit (Megabace) (Amersham) and applied to an automated DNA sequencing system (Megabace, Amersham). Sequences were analyzed using the Chromas software (Version 1.45, Griffith University, Qld, Australia). Comparison to the available sequences of the El Tor strain N16961 and classical strain O395 were done with the ClustalW 1.8 alignment program at the BCM Search Launcher: Multiple Sequences Alignment (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and NCBI-Blast (http://www.ncbi.nlm. nih.gov).

RESULTS

Presence of the nanH gene in the V. cholerae Amazonia genome - The presence of the *nanH* gene in the *V. cholerae* Amazonia 3509 genome was established by PCR amplification. A 1.1 kb fragment was obtained with primers *nanH*-f and *nanH*-r. The size of this fragment for the Amazonia strain is the same as the El Tor predicted product. In order to verify if other isolates of the Amazonia strain also carried the gene, amplification was carried out with isolates 3439, 3506, 4008, 4010 and 4132, all with positive results (data not shown).

Chromosomal location of the nanH gene of V. cholerae Amazonia - The position of the *nanH* gene was analyzed using the techniques of PFGE and DNA hybridization, making a comparison of Amazonia strain isolate 3509 and El Tor strain N16961. *Not*I was chosen as a first restriction enzyme to use, as *nanH* is in the center of a 172.5 kb *Not*I fragment in the El Tor strain, a size easily amenable to analysis. After PFGE, the gels were transferred to nylon membranes and hybridized to ^{32}P radioactive probes. The 1.1 kb internal *nanH* Amazonia fragment was used as a probe. A hybridization control experiment was done with a strip from the same gel shown, using a *toxT* internal fragment, obtained with primers *toxT* f59 and *toxT* r859, and known to be present in the El Tor strain but not the Amazonia strain.

Fig. 1a shows the *Not*I restriction digests of the Amazonia and El Tor strains DNA, and Fig. 1b shows the result of hybridization with the *nanH* probe. The expected 172.5 kb El Tor fragment showed hybridization. In the case of the Amazonia strain, a smaller, 150 kb fragment hybridized to *nanH*. Fig. 1c shows the hybridization to *toxT*, confirming that the Amazonia strain does not carry *toxT*, and detecting an 88.3 kb fragment for the El Tor strain, the size expected from the genome sequence.

Other enzymes were used, in order to make a better assignment of the gene position. Fig. 2a shows *Sfi*I DNA restrictions of strains Amazonia and El Tor and hybridization to the *nanH* probe. It can be seen that the upper *Sfi*I band of the Amazonia and El Tor strains hybridize to *nanH*. The Amazonia band with *nanH* was measured as 630 kb,

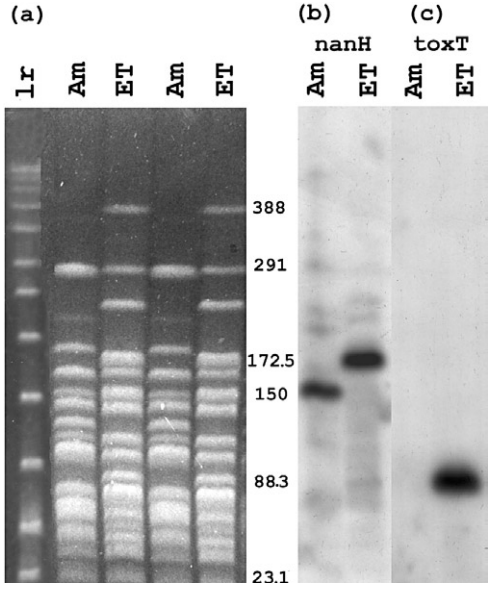

Fig. 1: pulsed-field gel and Southern blot analysis of *NotI*-digested chromosomal DNA of *Vibrio cholerae*. lr, low-range PFG markers, Am, Amazonia strain 3509, ET, El Tor strain N16961. Program 1 was used for the PFGE (a). The gel was cut in two strips, transferred to nylon membranes and these were used in hybridizations with probes for genes *nanH* or *toxT*, detecting the presence of *nanH* in both strains (b), and confirming the presence of *toxT* only in the El Tor strain (c). The sizes of the chromosomal fragments carrying *nanH* are 172.5 kb for the El Tor strain and 150 kb for the Amazonia strain, as seen in filter (b). The control strip (c) shows a fragment of 88.3 kb carrying the *toxT* gene in the El Tor strain, and no hybridization with the Amazonia chromosomes.

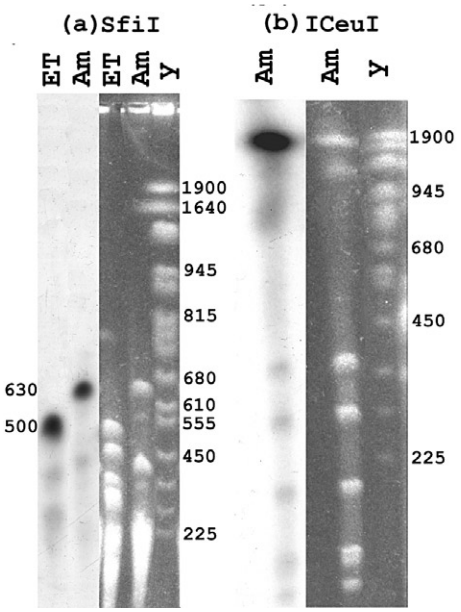

Fig. 2: pulsed-field gels and Southern blot analysis of *SfiI* or I-*Ceu*I-digested chromosomal DNA of *Vibrio cholerae*, estimating the size of fragments carrying the *nanH* gene. Am, and ET as in Fig. 1, y, yeast DNA-PFGE markers. (a) Pulsed-field gel (program 3) with *SfiI*-digested DNA on the right panel, and on the left the corresponding autoradiogram, showing fragments of 630 kb and 500 kb hybridizing with the *nanH* probe for the Amazonia and El Tor strains, respectively. (b) Pulsed-field gel of I-*Ceu*I-digested Amazonia strain DNA (program 2, right panel) and the hybridization of a 1900 kb fragment to the *nanH* probe (left panel).

and the El Tor band as 500 kb. The I-*Ceu*I DNA fragments were also hybridized to *nanH*. Fig. 2b shows that a large Amazonia DNA fragment carries the *nanH* gene, comparable in size to the El Tor 1900 kb corresponding fragment (El Tor data not shown).

Sequence of V. cholerae Amazonia nanH - The DNA sequence of *nanH* from isolate 3509 was determined. The 1.1 kb PCR fragment was used, but other regions were amplified in order to cover the whole gene. Primer pairs *LnanH*2814f and *LnanH*3626r, *LnanH*2407f and *LnanH*3766r, *nanH*1359f and *nanH*2262r, *nanH*2784f and *nanH*3473r, and *nanH*2838f and *nanH*3953r (Table II) yielded fragments of expected sizes (approximately 813pb, 1360pb, 921 bp, 707 bp, and 1133 bp, respectively), and allowed the sequencing of the whole *nanH* gene (2424 bp), and the next gene, similar to El Tor VC1785 (207 bp). The 3234 bp sequence has been deposited in the Genbank, with accession No. AY825932.

nanH sequence comparison - Jermyn and Boyd (2005) proposed a molecular evolution of VPI-2 based on a comparison of a 0.7 kb fragment of the *nanH* sequence of various strains. With the whole sequence of *nanH*, it was possible to extend this analysis in the case of the Amazonia strain. Table III (a and b) shows the nucleotide and amino acid substitutions found for this allele of the gene, in comparison to the sequence of VC1784 from the El Tor strain N16961 (Heidelberg et al. 2000). The sequence for classical strain O395 is identical (Galen et al. 1992), and these strains can be taken as representative of the pandemic strains. Seventy-two nucleotide substitutions were found, with an overall substitution rate of 2.97% along the 2424bp. They result in seventeen amino acid substitutions.

TABLE III

Nucleotide and amino acid sequence differences between El Tor and Amazonia strains

(a) Nucleotides

Position in DNA[a,b]	1 2 0	1 9 5	1 9 8	2 0 4	2 4 0	2 4 3	2 9 0	4 5 6	5 0 1	5 0 5	5 1 6	5 9 4	6 1 2	6 2 7	6 3 0	6 6 0	6 9 3	7 5 6	7 6 2	7 6 5	7 7 4	7 8 9	7 9 8	8 1 9	8 2 2	8 2 5	8 2 8	8 5 8	8 8 5	9 0 0	9 3 9	9 5 1	9 7 2	9 7 9	9 8 4	9 8 7
El Tor [c]	A	T	A	A	C	C	T	T	C	G	C	C	T	G	T	G	T	G	T	A	C	G	A	G	A	A	T	A	T	C	G	C	T	T	T	G
Amazonia	T	C	T	G	T	T	A	C	T	A	T	T	A	A	G	A	C	A	C	G	T	T	G	C	T	C	C	C	C	A	A	G	C	A	C	A
Codon [d]	3	3	3	3	3	3	2	3	3	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	1	3	3
Aa change [e]							*						*																							*

(cont.)

Position in DNA	1 0 0	1 1 0	1 1 0	1 1 5	1 1 5	1 1 7	1 1 9	1 2 5	1 2 5	1 2 5	1 2 8	1 2 9	1 3 2	1 3 3	1 3 4	1 3 4	1 3 5	1 3 5	1 3 7	1 3 8	1 4 9	1 4 3	1 4 3	1 4 6	1 4 6	1 4 7	1 4 7	1 5 3	1 5 5	1 5 6	1 5 7	1 5 8	1 6 1	1 6 1	1 6 7	1 8 8	1 9 5
El Tor	T	C	A	A	T	G	G	T	A	G	A	G	T	G	C	C	G	A	A	C	A	C	C	A	A	G	C	A	T	G	T	A	G	A	C	A
Amazonia	C	T	G	G	A	A	A	C	T	A	G	T	C	A	T	A	A	G	G	T	G	G	G	C	C	A	T	C	C	A	A	G	A	G	T	G
Codon	3	3	2	3	3	3	2	3	3	3	1	1	3	1	3	2	3	2	3	3	2	2	1	2	3	1	3	3	2	3	3	3	3	3	3	3
Aa change			*				*				*	*		*		*		*			*	*	*	*		*			*		*					

(b) Amino acids

Position in protein	9 7	1 6 9	3 2 7	3 6 9	3 9 9	4 2 8	4 3 3	4 4 2	4 5 2	4 6 0	4 7 8	4 7 9	4 8 8	4 9 3	4 9 9	5 1 1	5 2 2	5 2 7
El Tor	I	V	S	Q	S	S	V	G	A	H	H	T	Q	A	K	V	H	
Amazonia	N	I	T	R	N	G	F	S	D	R	R	S	A	T	N	A	Q	
Substitution type[f]	N	C	C	C	N	N	N	N	N	N	C	N	N	N	N	N		
Protein domain [g]	1	1	p	p	2	2	2	2	2	2	2	2	2	2	2	2	2	

a: numbers (in vertical format) correspond to nucleotide positions along the *nanH* gene in (a), and to amino acid positions along the NanH protein in (b), using the TIGR-CMR annotation (http://www.tigr.org/tigr-scripts/CMR2/GenePage.spl?locus=VC1784). The mature protein starts at amino acid 51 with this numbering. In order to make a correlation to the amino acid numbers in Crennel et al. (1994), decrease 26 from the given numbers in (b); *b*: the region analyzed in Jermyn and Boyd (2005) is marked by darker borders in both tables; *c*: El Tor strain N16961 and Amazonia strain 3509; *d*: positions in codon; *e*: nucleotide substitutions leading to changes in amino acids. Note that substitutions in positions 1462 and 1463 change a single amino acid; *f*: C, conservative, and N, non-conservative amino acid substitutions (BLOSUM 62 matrix, Henikoff & Henikoff 1992); *g*: NanH comprises three domains: 1, lectin wing 1; p, β-propeller; 2, lectin wing 2.

DISCUSSION

The analysis of whole genomes is leading to more insight into the flexibility of genomes, and their acquisition of a variety of genes that distinguish strains from one another, and may lead to an increased virulence of particular strains. In *V. cholerae* the two main virulence regions are examples of movable DNA, that may be acquired by other strains: the CTXΦ, a lysogenic phage carrying the genes for cholera toxin, and VPI (or VPI-1), carrying genes for the TCP pilus, involved in colonization. VPI-2 was described as a new pathogenicity island of toxigenic *V. cholerae*, carrying the *nanH* gene for neuraminidase (Jermyn & Boyd 2002). The presence of *nanH* in various isolates of the Amazonia strain, which is non-toxigenic, shows that this general association of *nanH* to toxigenic strains is not so strict. The DNA sequence obtained for *nanH*, with a 2.97% difference to the El Tor strain, rules out the possibility of a *nanH* horizontal transfer to the Amazonia strain during the Latin American epidemic of the 1990s.

NanH is known to have other cellular roles, such as sialic acid metabolism. Sialic acid is a source of carbon, nitrogen, energy and cell wall biosynthesis (Vimr & Troy 1985, Vimr et al. 2004). Another important role is as part of the mucinase complex. This complex contains neuraminidase, proteinases and an endoglycosidase (Stewart-Tull et al. 1986). The mucinase complex acts on the mucus gel protecting the underlying intestinal cells. The breakdown of sialomucin allows the bacteria to reach and colonize the epithelium.

V. cholerae Amazonia is a pathogenic strain, and these modulator or accessory virulence factors may be important for pathogenic non-toxigenic strains. There are several reports of local outbreaks of cholera caused by non-epidemic strains (Sharma et al. 1998, Cheasty et al. 1999, Pal et al. 1999). These are either O1 or non-O1 isolates, and many of these are non-toxigenic, posing a question about their virulence mechanism. The presence of additional virulence factors has been proposed. These include the non-membrane-damaging cytotoxin, Rtx toxin, hemolysins, proteases and haemagglutinins (Mitra et al. 1998, Cheasty et al. 1999, Lin et al. 1999). Colonization in itself could cause diarrhea (Kaper et al. 1994), and the presence of neuraminidase, helping colonization, may be one factor in this puzzle.

PFGE was done, to locate the *nanH* gene of *V. cholerae* Amazonia, comparing the position of the gene to its position in strain N16961. The N16961 strain in our laboratory originates from Dr Kaper's laboratory (University of Maryland), as is the case for the sequenced strain. Even so, we found a difference in the *Sfi*I fragments obtained, in relation to the fragments expected from the genome sequence. The larger *Sfi*I fragment found in our case is the 500 kb fragment, that hybridizes with *nanH* (Fig. 2a), and also to *rtxA* and *ctxA* (data not shown). We propose that one *Sfi*I site that should be present at position 1625927 of the genome is not present in our strain, and two adjacent fragments of 89 kb and 411 kb are joined together as the largest fragment of *V. cholerae* El Tor. This El Tor *Sfi*I fragment is located inside the largest 1900 kb I-*Ceu*I fragment. For the Amazonia strain a I-*Ceu*I fragment of similar gel mobility was found. A precise size assignment is difficult to make for fragments of this size. The presence of *nanH* into this fragment, larger than chromosome 2, places *nanH* into chromosome 1. In a preliminary genome map of the Amazonia strain that we are constructing, the fragments carrying *nanH* in the Amazonia genome, I-*Ceu*I of 1900 kb, *Sfi*I of 630 kb and *Not*I of 150 kb, correspond to the El Tor fragments of 1900 kb (I-*Ceu*I), 500 kb (*Sfi*I) and 172.5 kb (*Not*I), respectively. The conclusion is that the position of *nanH* in the Amazonia strain genome is in the same region as in the El Tor strain.

NanH is a large neuraminidase (83 kDa), a three-domain protein consisting of two lectin wings and a central active neuraminidase domain, formed by six β-sheets arranged as in the blades of a propeller (β-propeller, Crennel et al. 1994, Moustafa et al. 2004). In studies with crystals of the classical strain O395 neuraminidase, some amino acids were proposed to be relevant for activity (Crennel et al. 1994), and all of these are conserved in the Amazonia strain. They include Arg250 (see footnote a to Table III concerning the numbering system used), Arg661, Arg738, Glu782, Tyr766, Glu645, Trp337, Asp276, Arg271, Asp318, Asp663, Asn344, Phe664, and Leu606. The Asp-boxes (Ser/Thr-X-Asp-[X]-Gly-X-Thr-Trp/Phe) of the β-propeller (Crennel et al. 1994) are also conserved in the Amazonia sequence.

Seventeen amino acid substitutions were found in NanH, in comparison to the identical sequences of El Tor strain N16961 and classical strain O395 (Table IIIb). Thirteen of these are in the second lectin wing of the neuraminidase, the most variable region of the protein for the Amazonia strain. The first lectin wing is known to bind sialic acid, but the ligand for the second wing is unknown (Moustafa et al. 2004). The first wing of the Amazonia NanH presents only two substitutions, one of these a conservative substitution in one of the 15 β-strands of the domain, and the other in a loop region. In the β-propeller active domain of the enzyme there are two conservative substitutions, one of these in one of the 24 β-strands of this domain. In the second lectin wing, comprising 15 β-strands and 2 α-helices, 6 of the substitutions are in β-strands (1 conservative, 5 non-conservative), 1 in an α-helix (non-conservative), and 6 in connection loops.

Jermyn and Boyd (2005) used a 0.7 kb PCR fragment from various strains of *V. cholerae* and *V. mimicus*, in a study of variation of *nanH*. A comparison of our data to the corresponding stretch analyzed in that paper shows that the Amazonia strain presents an allele 2 for *nanH*, identical, in this region, to the allele in strain E714. The description given in that paper for this strain is incomplete, and its serogroup and isolation date are not known. The other strain with an allele 2 belongs to serogroup O8. The O1 and O139 strains studied all carry an allele 1. The Amazonia strain is the first O1 strain with a new allele for *nanH*.

Taking into consideration our results for *V. cholerae* Amazonia, we propose a shift in the region of *nanH* to be analyzed and compared in future allele studies. Nucleotides 1105 to 1581 encompass fourteen amino acid substitutions for *V. cholerae* Amazonia, as compared to only two changes in the region analyzed for various strains (approximately 264 to 858, Jermyn & Boyd 2005).

In order to sequence the whole *nanH* gene, we had to design primers located outside *nanH*, in nearby VPI-2 regions according to the El Tor N16961 sequence. The amplifications worked well, yielding fragments with the expected sizes. The gene located immediately to the right of *nanH* in the El Tor strain, VC1785, was found in the same position in the Amazonia strain. Based on the presence of the *nanH* gene and nearby VPI-2 regions, we propose that the Amazonia strain carries a VPI-2 region, to be detailed in further studies.

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