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Correlation between Detection of a Plasmid and High-Level Virulence of *Vibrio nigripulchritudo*, a Pathogen of the Shrimp *Litopenaeus stylirostris*

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Abstract:

Vibrio nigripulchritudo, the etiological agent of *Litopenaeus stylirostris* summer syndrome, is responsible for mass mortalities of shrimp in New Caledonia. Epidemiological studies led to the suggestion that this disease is caused by an emergent group of pathogenic strains. Genomic subtractive hybridization was carried out between two isolates exhibiting low and high virulence. Our subtraction library was constituted of 521 specific fragments; 55 of these were detected in all virulent isolates from our collection (*n* = 32), and 13 were detected only in the isolates demonstrating the highest pathogenicity (*n* = 19), suggesting that they could be used as genetic markers for high virulence capacity. Interestingly, 10 of these markers are carried by a replicon of 11.2 kbp that contains sequences highly similar to those of a plasmid detected in *Vibrio shilonii*, a coral pathogen. The detection of this plasmid was correlated with the highest pathogenicity status of the isolates from our collection. The origin and consequence of this plasmid acquisition are discussed.

1. Introduction

Vibriosis are important diseases threatening the sustainable development of the penaeid shrimp aquaculture industry (6, 7, 20, 21). However, little research attention has been paid to these diseases, mostly because viruses are considered as the most significant diseases in crustacean aquaculture (22). As a result solutions to control vibriosis remain scarce. The massive use of antibiotics is certainly not a sustainable control strategy since it favors emergence of antibiotic resistant strains (17). Moreover, antibiotics are often banned from aquaculture ponds for commercial reasons and because of the increasing concern for residue issues. Additionally, vaccination is not possible in invertebrates thereby limiting the ways to reduce disease impact in shrimp culture (2). Therefore, there is a need to understand the environmental, physiological and bacterial conditions leading to the expression of a disease in order to determine ecological or zootechnical methods that could in the end control these diseases (16). One step consists in diagnosing and quantifying the etiologic agent of a disease, both in the reared animal and in its environment.

It is recognized that strains belonging to a same *Vibrio* species can have different virulence patterns ranging from highly pathogenic strains to non-virulent ones (8, 13, 19). Therefore, the diagnosis often needs to be infraspecific i.e. based on epidemiologically relevant sequence polymorphisms that can be regarded as genetic markers of virulence (9, 25).

Suppressive Subtractive Hybridization (SSH) has been successfully and extensively used in a wide range of bacterial species to identify strain-specific genes (14, 32, 33). In case of virulent versus non-virulent strains comparison, the differential genes evidenced may encode for virulence factors. Moreover, identifying these strain-specific regions can help to bring to evidence the trace of horizontal gene transfers (HGT) that are known to provide selective advantages and to be implicated in the emergence of new pathogen (4, 10, 24).

In New-Caledonia (South Pacific, 19°S/23°S), a disease called “summer syndrome” has occurred seasonally in penaeid shrimp farms since 1997 and causes severe epizootic mortalities. A multidisciplinary research program aiming at a global understanding of this disease was set up, bringing together rearing technology, pond ecosystem studies, shrimp physiology and immunology, nutrition and genetics, pathology and bacteriology approaches (11). Epidemiological studies have revealed that this disease is a vibriosis due to highly pathogenic *Vibrio nigripulchritudo* isolates (7, 8). To the best of our knowledge, this is the first reported disease associated with this V*ibrio* species. Because the New Caledonian shrimp production is also affected by another vibriosis, namely syndrome 93 occurring during the cool season (6), the spreading of the summer syndrome to other shrimp farms would undoubtedly threaten the sustainable development of the New Caledonian shrimp industry. Preliminary studies based on a collection of *V. nigripulchritudo* isolates have brought to light different virulence levels according to experimental infection results (7). The genetic structure of 58 *V. nigripulchritudo*-selected isolates was then studied using Arbitrarily Primed PCR (AP-PCR) and Multilocus Sequence Typing (MLST) (8). These two typing methods gave congruent results revealing a clustering of highly pathogenic (HP) and moderately pathogenic isolates (MP). None of the non pathogenic (NP) isolates were present in this cluster. The hypothesis of the emergence of this particular cluster of pathogenic *V. nigripulchritudo* within a shrimp farm environment has been proposed. This emergence could be linked to the recent acquisition of one or several genetic elements leading a moderately virulent isolate to become highly pathogenic.

Our study was aimed at identifying and characterizing genetic markers of *V. nigripulchritudo* virulence by a Suppressive Subtractive Hybridization performed between the genomes of a HP isolate and a genetically close, NP isolate. In a second step, the distribution of the screened SSH fragments was studied in a selection of both virulent (either HP or MP) and NP *V. nigripulchritudo* isolates by macro-array. This allowed us to determine more precisely which DNA fragments are constantly associated with the virulence and could possibly be part of the virulence determinants. Lastly, the discovery of a replicon detected only in HP *V. nigripulchritudo* isolates, leads to a discussion of the role of mobile genetic elements in the emergence of pathogenicity in *V. nigripulchritudo.*

2. Material and methods

2.1. Bacterial strains media and DNA extraction

The *V. nigripulchritudo* isolates used in this study have been described previously and are presented in Table 1 (8). The *V. shilonii* strain AK1 was purchased from the Pasteur Institute collection (CIP107136T). *Vibrio* strains were grown in marine broth (MB) or marine agar (MA) at 30°C. *E. coli* strains were routinely grown in Luria-Bertani (LB) at 37°C. All media were from Difco (http://www.difco.com). When necessary, media were supplemented with ampicillin (100 µg/ml). Total genomic DNA from *Vibrio* strains was prepared as described previously (30).

2.2. Polymerase chain reaction (PCR)

Long range PCR was performed using Herculase DNA polymerase fusion II to amplify the entire plasmid of *V. nigripulchritudo* or *V. shilonii* following the manufacturer's instructions (http://www.stratagene.com). Other PCR reactions were performed using the Bioline Taq polymerase according to the manufacturer's instructions. Conditions for amplification were as follows: 94^{o}C for 3 min, followed by 30 cycles of 94^{o}C for 30 s, (Tm-10°C) for 30 s, and 72^{o}C for 60 s per kbp.

2.3. Suppression subtractive hybridization and macroarrays

SSH was carried out using the PCR-Select Bacterial Genomic Subtraction Kit (**http://www.clontech.com/**) essentially following the manufacturer's instructions. Isolate SFn1 (HP) genomic DNA was used as the tester and SFn118 (NP) genomic DNA was used as the driver DNA. During SSH, a high annealing temperature of 63°C was used to enrich for the recovery of SFn1-specific unique sequences. The PCR products obtained from SSH, representing tester-specific sequences, were cloned into pCRII-TOPO (**http://www.invitrogen.com/**) and transformed into *E. coli* strain TOP10.
Recombinant clones were screened by macro-arrays. Briefly, inserts were PCR amplified and spotted in duplicate onto nylon membranes (http://www.millipore.com/). Genomic DNAs were labeled and used as probe in hybridization experiments using the DIG-labeling and detection kit according to the manufacturer instruction (http://www.rochediagnostics.fr/).

2.4. DNA sequencing and sequences analysis

DNAs to sequence were amplified using a Templi Phi Amplification Kit (**http://www.amersham.com/**) and sequenced on an ABI PRISM 3100 Genetic Analyzer (**http://www.appliedbiosystems.com/**) following manufacturer instructions. DNA sequences were blasted on public databases using blastX algorithm (1). Similarities with an E-value smaller than 10^{-5} were considered significant. Sequencing, contig assemblies were performed by using SEQMAN (Lasergene software).

2.5. Plasmid extraction and characterisation

Plasmidic DNA extractions trials were conducted using different commercial kits, namely Qiafilter Plasmid Midi kit (http://www1.qiagen.com/), Plasmid Midiprep kit (http://www.sigmaaldrich.com) and Wizard DNA Purification System (http://www.promega.com/) following the instructions of the manufacturers.
Plasmids were digested with restriction enzymes *Eco*RI and *Xho*I, size fractionated by 1% agarose electrophoresis and analyzed by Southern blot (30). For this, a fragment of the plasmid pSFn1 (SSH clone 16) was PCR amplified and labeled using the Dig Labeling system (Roche).
Complete sequences of pSFn1 and pAK1 were obtained by shotgun sequencing. After *Sau*IIIa partial digestion the purified 3 kbp to 8 kbp restriction DNA fragments were ligated in a pUC18 vector predigested by *Bam*HI (Amersham) and transformed into TOP10 competent cells (Invitrogen). ORFs annotation was performed using GeneMark software and syntheny analysis using Arthemis software.

2.6. Nucleotide sequence accession numbers

The DNA sequences of the plasmid pSFn1 and pAK1 have been assigned the accession number respectively EU156059 and EU159455.

3. Results

3.1. Genomic subtraction between *V. nigripulchritudo* isolates

A Suppressive Subtractive Hybridization was carried between the HP isolate SFn1 and the NP isolate SFn118. In order to check the specificity of the technique, a total of 1112 inserts from the SSH library was screened by macro-array using SFn1 or SFn118 genomic DNA as probe. 622 SFn1-specific fragments were selected revealing 44.1% of non-specific DNA fragments. Sequencing of the SFn1 specific fragments resulted in 521 DNA sequences among which 143 (27.4%) showed no significant matches with entries in the public database. The remaining 378 predicted ORFs showed homology to proteins described in other bacterial species, and among them 43 (18.9%) corresponded to conserved hypothetical proteins.

3.2. Correlation between macroarrays and virulence status

In a first set of experiments, macro-arrays were performed using the 521 SFn1-specific DNA fragments as targets and a collection of 19 *V. nigripulchritudo* genomic DNA as probes. This allowed the selection of 68 DNA fragments: 13 were found only in the DNA of the HP isolates whereas 55 were present in both HP and MP isolates (n=5 and 6 isolates, respectively). The 8 NP isolates genomic DNA hybridized with almost none of the 68 selected DNA fragments. In a second set of experiments, the 68 fragments specific to the pathogenic isolates (both HP and MP) were spotted on membranes that were then hybridized with genomic DNA extracted from 33 additional *V. nigripulchritudo* isolates. Hybridization profiles were correlated with virulence status i.e. 13 fragments were found specific to the 19 HP isolates, 55 fragments were found in HP and in MP isolates and only a few fragments (n=23) were found sporadically in NP isolates (Fig. 1).
Among the 68 putative ORFs inferred, the homology search suggests that some could play a role in the virulence process (Table 2). Clone 106 presents similarity to a vulnibactin outer membrane receptor precursor, clone 198 to an RTX protein or autotransporter adhesin, clone 458 to cyanobacterial toxins and clone 486 to a capsule biosynthesis protein CapA. Nine putative ORFs (13%) showed homology with transposase, integrase or other proteins implicated in recombination suggesting a role of mobile elements in the SFn1 genome specificity.

3.3. Identification and genetic organization of the pSFn1 plasmid

Within the subgroup of SSH fragments detected only in HP isolates, 3 clones (clone 16, 68 and 155) contained a partial ORF with high similarity to two genes (Z2Z3 and Z8) found in one of the plasmids evidenced in *V. shilonii* (Rosenberg *et al*., unpublished data).
Previous experiments using the protocol described by Kado and Liu (15) failed to demonstrate the presence of a plasmid in *V. nigripulchritudo* isolates. Since the detection of fragments Z2Z3 and Z8 suggested the presence of a plasmid in SFn1, three additional extraction protocols were tested; only the Qiafilter Plasmid Midi Kit (Qiagen) allowed purification of a replicon from this isolate.
The complete sequence of the replicon named pSFn1 (11237 bp) was obtained and the putative ORFs identified using GeneMark software. A graphical representation of the 10 predicted ORFs appears in the Figure 2. Their relationships to their homologues in databases are detailed in Table 2. Five ORFs showed significant similarity with known genes coding for: a putative partitioning protein (ORF2) a putative phage tail protein (ORF4), a phage head-tail tape measure protein (ORF5), a serine peptidase S49 family (ORF6) and an activator of ProP osmoprotectant transporter (ORF10). Two ORFs correspond to conserved hypothetical protein (ORF3 and 8) and three are unknown (ORF1, 7 and 9).
Among the 13 DNA fragments that were demonstrated to be present in all HP isolates, 10 were localized in this plasmid pSFn1.

3.4. Correlation between plasmid and virulence

The successful plasmid extraction procedure was conducted with a larger panel of isolates. A single plasmid was evidenced in 4/4 additional HP isolates (SFn27, SFn135, POn19, POn3). In 1/8 MP (AQn1) and 1/7 NP (AgMn1) isolates one or more plasmid(s) were also purified.
RFLP analysis of the plasmids was performed using *Eco*RI or *Xho*I restriction enzymes and demonstrated that these 4 HP isolates harbor a plasmid identical or very similar to pSFn1 with 3

*Eco*RI restriction fragments (1.1, 3.4, and 6.6 kbp) and one *Xho*I linearized plasmid of 11.2 kbp. In case of isolates AQn1 and AgMn1 *Eco*RI and *Xho*I plasmid restriction profiles were found to be clearly distinct (data not shown). Furthermore double digestions suggest a single larger plasmid or the existence of several plasmids. Results were confirmed by southern blot using SSH Fragment 16 as probe. An *Eco*RI digested fragment of 3.4 kbp (in pSFn1) was evidence only in all tested HP isolates.

3.5. Comparison between pSFn1 and the plasmid pAK1 of *V. shilonii*

The same plasmidic extraction procedure was used successfully to purify plasmid from *V. shilonii* strain AK1. In agreement with Rosenberg *et al*. (unpublished data) more than one plasmid was obtained. Among several primers designed on the basis of the pSFn1 sequence, primers 9F and 9R, localized between ORF5 and 6, were successfully used to amplify by inverse PCR a fragment of 13.4 kbp which was further sequenced to provide the complete sequence of one of the AK1 plasmids, named pAK1. As for pSFn1, the putative ORFs were identified using GeneMark software.
A DNA:DNA comparison between plasmids showed that 71.8% of pSFn1 was shared with pAK1 , with 93% of nucleotidic identity for these sequences (Fig. 3). Syntheny analysis highlight that 5 regions were significantly similar between pSFn1 and pAK1 (score>500).

4. Discussion

Compared to human bacterial pathogens, little is known concerning *Vibrio* pathogenesis in marine invertebrates. The genetic diversity of *Vibrio* as well as the complexity of virulence mechanisms causes difficulties in diagnosing vibriosis. Among approaches that can be proposed to investigate genetic markers of pathogenicity
Whole genome sequencing appears as the most informative approach as it has significantly improved our understanding of the physiology and pathogenicity of many microbes and provided insights into the mechanisms and history of genome evolution (5). The genomes of four *Vibrio* species have already been sequenced: *V. cholerae* (12), *V. parahaemolyticus* (23), *V. vulnificus* (3), and *V. fischeri* (29). This makes comparative genomics an attractive approach to investigate the basis of virulence in *Vibrio*. However, in spite of recent progress in high density sequencing method, this approach is still laborious and expensive and as a consequence is restricted to a limited number of strains. Furthermore, if whole genome sequencing allows to rapidly and extensively hypothesize virulence mechanisms based on putative virulence determinants deduced from known functions of heterologous or orthologous known sequences, the functional demonstration of their involvement in virulence would still rely on mutagenesis and complementation of the candidate genes.
Subtractive hybridization methods are techniques designed to identify genomic regions that are present in one genome but absent from another (32). The application of such a method has led to the identification of genomic islands (26), mobile genetic elements (31) and plasmids (18). In case of virulent versus non-virulent strain comparison, such regions could correspond to virulence genes or regulators. Therefore this relatively simple and cheap technique is attractive to investigate genomic variation and identify virulence factors.
In a former study, a collection of *V. nigripulchritudo* isolates was studied in order to gain a better understanding of the epidemiology of this pathogen in New Caledonia (7, 8). Bacteria phenotypically related to this species were isolated from shrimps suffering “Summer Syndrome” or from other contexts and over a wider geographical coverage (Table 1). Molecular typing using two different techniques, Arbitrarily Primed PCR (AP-PCR) and Multi Locus Sequence Typing (MLST) were congruent and permitted the definition of a cluster which included all “Summer Syndrome” isolates from diseased animals, from the two affected farms whatever their date of isolation. Together with these isolates, a few environmental isolates from the affected farms (sediment or pond water) suggest that they may be environmentally transmitted.
By experimental infection the isolates of this cluster were demonstrated to be moderately to highly pathogenic. This sum of data led us to hypothesize that the Summer Syndrome is attributable to a single pathogenic clone, surviving from one year to the next in the shrimp farm environment then re-developing inside the grow out system at the next crop.
The correlation between virulence phenotype and taxonomic markers suggests that virulence genes, at least in part, are carried by one of the two chromosomes. However because genotyping studies do not allow distinguishing HP isolates from MP ones, more recent genetic events can be suspected to

be at the origin of HP isolate emergence inside this cluster. Such a recent evolution often implies mobile elements that can be tracked by the SSH approach.
In the present study, the SSH approach comparing an HP versus an NP isolate allowed to identify 13 fragments specific for the HP isolates. Among these fragments 10 corresponded to putative ORFs harbored by a plasmid, pSFn1, evidenced only in the HP isolates and showing high similarities with a 13.5 kbp plasmid described in *V. shilonii* (Rosenberg et al., unpublished data). This latter *Vibrio* was also putatively identified, together with *V. nigripulchritudo*, in corals along the coasts of Florida (27) suggesting that *V. nigripulchritudo* and *V. shilonii* can coexist in a same ecological niche.
Our hypothesis is that coral or shrimp**,** as other marine invertebrate with the millions of resident bacteria that are concentrated in their different compartments, as a niche, could be a suitable place for horizontal gene transfer. The exchange could impact different adaptive functions, leading to the capacity to colonize different ecological niches and ultimately the emergence of a specific clone. Therefore coral, shrimp or other invertebrates, could be at the origin of plasmid transfer.
V. shilonii has been associated with coral bleaching events in *Oculina patagonica* in the Mediterranean Sea. Many data concerning the temperature-regulated mechanism of infection, virulence mechanisms and pathogen transmission have been obtained experimentally with the strain AK1 (28). However no data concerning the epidemiological survey in situ are available. As a consequence the absence of results concerning the identification of plasmids within a collection of V. shilonii prevents a discussion of the role of this plasmid in the virulence of V. shilonii.
Here the presence of the plasmid pSFn1 has been clearly correlated with the HP status of *V. nigripulchritudo* isolates suggesting that this element had played a role in the HP emergence.
One hypothesis is that this plasmid harbors one or more genes involved in bacterial virulence and could be considered as a plasmid linked to virulence. However because no ORFs annotated in the plasmid can be clearly assigned to a pathogenicity factor, genetic approaches should be developed to investigate the role of this plasmid in virulence: pSFn1 curing from HP isolate, pSFn1 transferring to NP/MP isolates and ORFs deletion require experimental development that are currently in progress.
Furthermore the identification of 3 SSH fragments, HP specific, and absent from the plasmid, suggests that several virulent determinants are chromosomally localized. Further knock out strategies should target genomic virulence markers.
Hybridization analysis using 68 SSH-derived fragments appears more discriminating than MLST or AP-PCR because it allows the distinction of the HP from the MP isolates. Our results could lead in the future to the development of relevant tools for diagnosis of HP isolates, for instance a plasmid-specific PCR, thereby avoiding the need to characterize virulence by experimental infection. Those operational tools will allow evaluating the impact of this vibriosis on shrimp aquaculture in New Caledonia.

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Figures

Figure 1

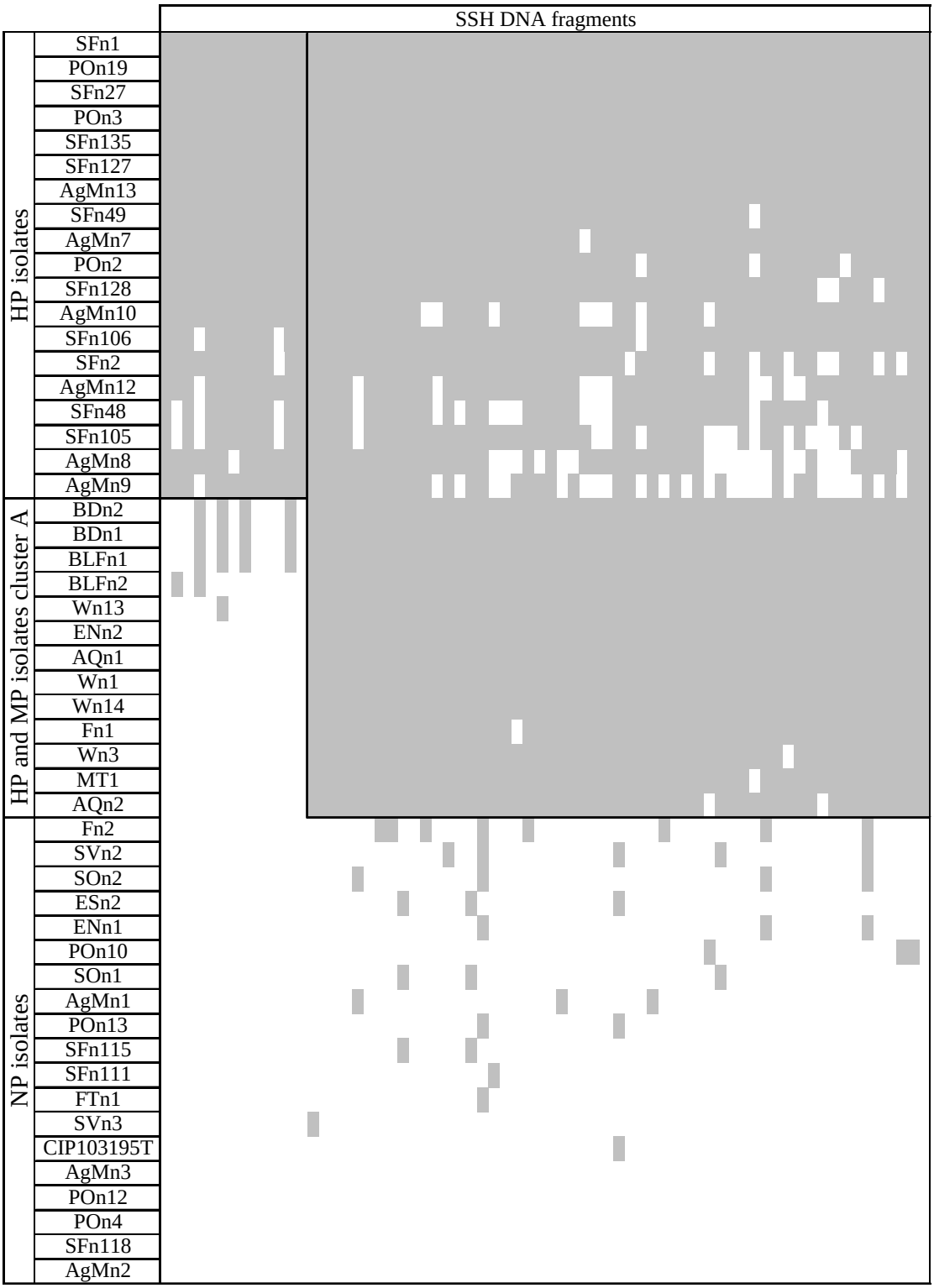

FIG. 1.
Correlation between subtracted fragments of genomic DNA from *V. nigripulchritudo* SFn1 and virulence statuses, summary of macro-array results. Hybridizations were performed using the 68 SFn1-specific DNA fragments as target and a collection of 51 *V. nigripulchritudo* DNA as probes. Name and virulence status of strains are indicated in ordinate, DNA subtracted fragment are indicated in abscissa in the same order than in the Table 1. Positive signals are indicated in grey.

Figure 2

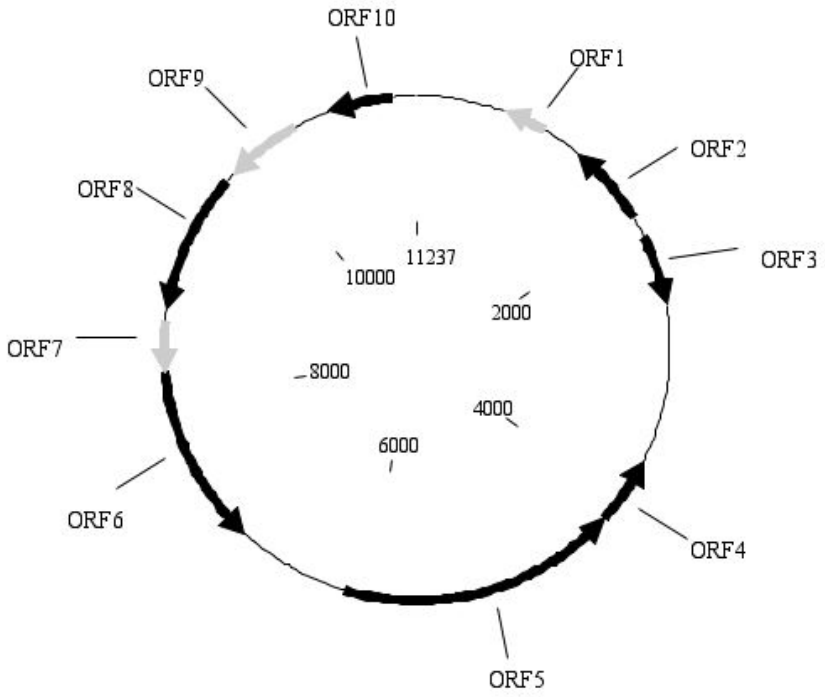

FIG. 2.
Open reading frames map of the 11237 bp plasmid pSFn1. The orientation of the putative ORFs is indicated by the orientation of the arrows; in black, ORFs with significant sequence similarities with BlastX algorithm on GenBank; in grey, putative ORFs for which no significant similarity was found.

Figure 3

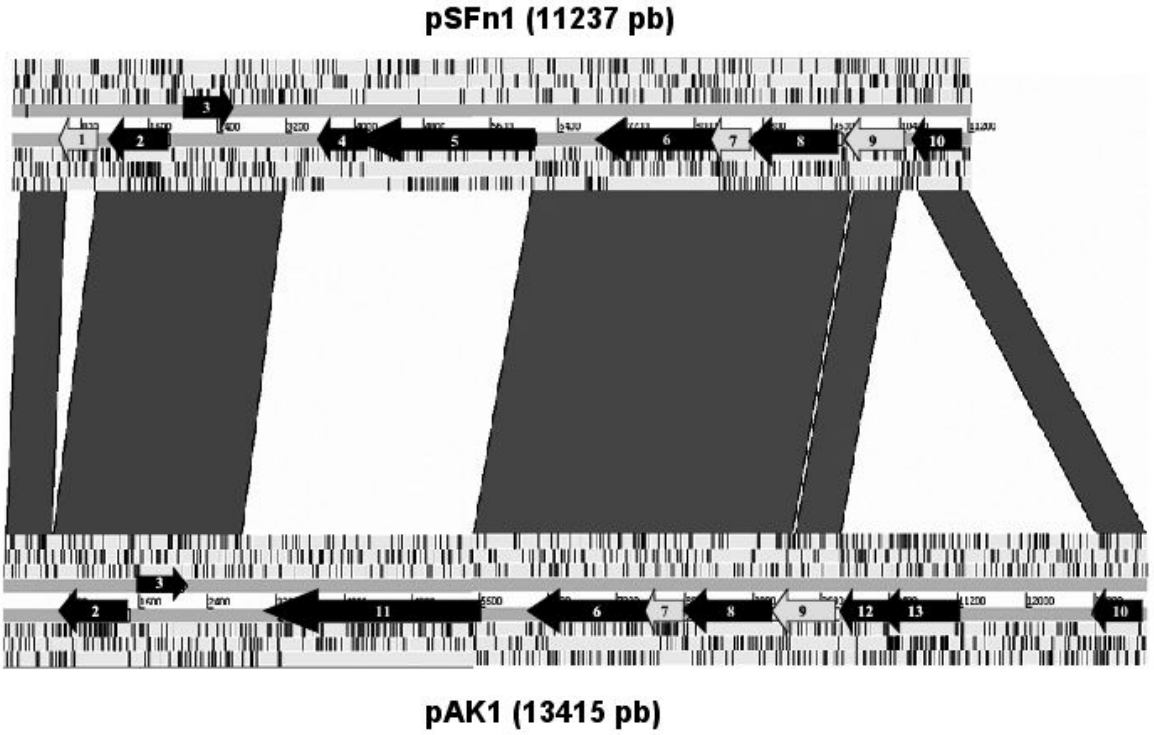

FIG. 3.
Linear comparison of pSFn1 and pAK1 plasmids. The ORFs of the two strands are indicated by grey arrows when no significant blast were obtained, and black arrows when significant blast were obtained. ORF2 encodes an ATPase involved in partitioning protein, ORF 4 and 5 encode a phage tail tape measure protein TP901, ORF6 encode a serine peptidase S49 family, ORF10 encode an activator of ProP osmoprotectant transporter, ORF11 encode a putative tail length determinator, ORF3, 8, 12 and 13 encode conserved hypothetical protein, ORF1, 7 and 9 encode an unknown hypothetical protein. The grey lines between the plasmid represent DNA:DNA similarities (BlastN matches between the two sequences, score>500).

Table 1

Strain name	context	Virulence to *L. stylirostris*
CIP 103195T	*V. nigripulchritudo* type strain	NP
SFn1	Summer syndrome, moribund shrimp hemoculture	HP
SFn2	Summer syndrome, moribund shrimp hemoculture	HP
SFn27	Sediment pore water, diseased pond	HP
SFn48	Summer syndrome, moribund shrimp hemoculture	HP
SFn49	Growout pond water, diseased pond	HP
SFn105	Growout pond water, diseased pond	HP
SFn106	Summer syndrome, moribund shrimp hemoculture	HP
SFn111	Carapace of a healthy crab (*Portunus pelagicus*), diseased farm	NP
SFn115	Lagoon water in front of pumps, diseased farm	NP
SFn118	Lagoon water in front of pumps, diseased farm	NP
SFn127	Healthy shrimp hemoculture, before disease outbreak	HP
SFn128	Summer syndrome, moribund shrimp hemoculture	HP
SFn135	Growout pond water, diseased pond	HP
AgMn1		NP
AgMn2	Healthy shrimp hemoculture, before disease outbreak (same animal)	NP
AgMn3		NP
AgMn7	Healthy shrimp hemoculture, before disease outbreak	HP
AgMn8	Summer syndrome, moribund shrimp hemoculture	HP
AgMn9	Growout pond water, diseased pond	HP
AgMn10	Summer syndrome, moribund shrimp hemoculture	HP
AgMn12	Sediment pore water, diseased pond	HP
AgMn13	Sediment pore water, diseased pond	HP
POn2	Healthy shrimp hemoculture, healthy pond 2, healthy farm	HP
POn3	Healthy shrimp hemoculture, healthy pond 3, same healthy farm	HP
POn4	Healthy shrimp hemoculture, healthy pond 6, same healthy farm	NP
POn10	Moribund shrimp hemoculture, no vibriosis, healthy pond 5, same healthy farm	NP
POn12	Healthy shrimp hemoculture, healthy pond 4, same healthy farm	NP
POn13	Healthy shrimp hemoculture, same healthy pond 4, same healthy farm	NP
POn19	Healthy shrimp hemoculture, same healthy pond 4, same healthy farm	HP
SOn1	Moribund shrimp hemoculture, no vibriosis	NP
SOn2	Healthy shrimp hemoculture, healthy pond, healthy farm	NP
FTn1	Moribund shrimp hemoculture, no vibriosis	NP
SBn2	Healthy shrimp hemoculture, healthy pond, healthy farm	NP
Wn1	Moribund shrimp hemoculture, opportunistic vibriosis	MP
Wn3	Moribund shrimp hemoculture, opportunistic vibriosis	MP
Wn13	Moribund shrimp hemoculture, opportunistic vibriosis (same animal)	MP
Wn14		MP
BDn1	Healthy shrimp hemoculture, healthy pond, healthy farm	MP
BDn2	Healthy shrimp hemoculture, healthy pond, healthy farm	MP
Fn1	Healthy shrimp hemoculture, healthy pond, healthy farm	MP
Fn2	Healthy shrimp hemoculture, healthy pond, healthy farm	NP
AQn1	Healthy shrimp hemoculture, healthy pond, healthy farm	MP
AQn2	Healthy shrimp hemoculture, healthy pond, healthy farm	MP
MT1	Moribund shrimp hemoculture, opportunistic vibriosis, broodstock	MP
BLFn1	Moribund shrimp hemoculture, opportunistic vibriosis	MP
BLFn2	Moribund shrimp hemoculture, opportunistic vibriosis	MP
ENn1	Healthy shrimp hemoculture, healthy broodstock	NP
ENn2	Healthy shrimp hemoculture, healthy broodstock	MP
SVn2	Moribund shrimp hemoculture, no vibriosis	NP
SVn3	Healthy shrimp hemoculture, healthy farm	NP
ESn2	Healthy shrimp hemoculture, healthy broodstock	NP

Table 1. *V. nigripulchritudo* strains and field isolates used in the present study. In bold: isolates from farms, which are affected by the Summer Syndrome. In italics: isolates collected during surveys specifically dedicated to the isolation of *V. nigripulchritudo* strains. Virulence as determined in text: HP stands for highly pathogenic strain, MP for moderately pathogenic and NP for non pathogenic.

Table 2

	SSH clone	Genbank accession N°	SSH DNA fragment size (bp)	predicted protein (bp)	homology	blast	E value	identity (%)	homologue accession N°
specific DNA fragments of the HP strains	16=ORFpSFn1 N°6	ET024018	459	990	13.5 kbp plasmid sequence Z2Z3, putative serine peptidase S49 [*Vibrio shilonii* AK1]	n	9E-175	92	AAB65791
	64	ET023962	427	1380	hypothetical protein VV0144 [*Vibrio vulnificus* YJ016]	x	6E-76	97	NP_932937
	68=ORFpSFn1 N°7	ET024019	250	553	13.5 kbp plasmid sequence Z8	n	7E-57	94	AF009903
	104	ET023965	415	2691	hypothetical protein Neut_2547 [*Nitrosomonas eutropha* C71]	x	2E-48	72	ABI60750
	155=ORFpSFn1 N°7	ET024020	312	553	13.5 kbp plasmid sequence Z8 [*Vibrio shilonii* AK1]	n	5E-86	88	AF009903
	191=ORFpSFn1 N°4	ET024021	323	1788	predicted phage tail protein [*Vibrio vulnificus*]	x	2E-05	45	ABB90701
	284=ORFpSFn1 N°8	ET024022	262	1035	hypothetical protein R2601_22861 [*Roseovarius sp.* HTCC2601]	x	5E-04	55	ZP_01444696
	302=ORFpSFn1 N°5	ET024023	367	2280	Phage tail tape measure protein TP901, core region [*Thiomicrospira crunogena* XCL-2]	x	4E-16	40	YP_390968
	378	ET024024	277	_	Unknown COG	x	_	_	_
	414	ET024025	537	_	Unknown COG	x	_	_	_
	506	ET024007	405	909	Predicted transcriptional regulator [*Vibrio alginolyticus* 12G01]	x	2E-05	47	ZP_01259885
	522=ORFpSFn1 N°5	ET024026	405	1815	putative tail length determinator [Bacteriophage K139]	x	2E-13	35	NP_536663
	535=ORFpSFn1 N°3	ET024027	365	489	hypothetical protein pC46022_21, Vibrio vulnificus	x	3,00E-33	53	YP_001393180
specific DNA fragments of all the strains of the cluster A	39	ET023960	187	_	Unknown COG	x	_	_	_
	55	ET023961	416	765	tsaC, RSc2351; probable toluenesulfonate zinc-independent alcohol dehydrogenase oxidoreductase protein [*Xanthobacter autotrophicus* Py2]	x	6E-15	63	ZP_01196427
	73	ET023963	429	1080	putative signal peptide protein [*Marinomonas sp*. MED121]	x	9E-42	59	ZP_01077543
	86	ET023964	331	1227	probable tartrate dehydrogenase/ 3-isopropylmalate dehydrogenase [*Rhodococcus sp.* RHA1]	x	1E-18	60	YP_708005
	106	ET023966	255	2061	**vulnibactin outer membrane receptor precursor [*Vibrio vulnificus*]**	x	3E-15	56	AAF28471
	116	ET023967	322	1143	hypothetical protein VP1567 [*Vibrio parahaemolyticus* RIMD 2210633]	x	1E-06	68	NP_797946
	129	ET023968	344	_	Unknown COG	x	_	_	_
	130	ET023969	366	906	ferrous iron efflux protein F [*Vibrio cholerae* O1 biovar eltor str. N16961]	x	6E-44	76	NP_232318
	135	ET023970	219	1038	iSSod13, transposase [*Vibrio vulnificus* YJ016]	x	1E-28	94	NP_934531
	154	ET023971	485	2058	ATPase involved in DNA repair-like protein [*Shewanella frigidimarina* NCIMB 400]	x	3E-41	70	YP_750769
	166	ET023972	375	_	Unknown COG	x	_	_	_
	173	ET023973	365	_	Unknown COG	x	_	_	_
	176	ET023974	347	3840	hypothetical protein CburD_01002029 [*Coxiella burnetii* Dugway 7E9-12]	x	3E-12	45	ZP_01298115
	196	ET023975	389	1539	Phage integrase [*Thiomicrospira crunogena* XCL-2]	x	1E-17	44	YP_390599
	197	ET023976	372	1038	iSSod13, transposase [*Vibrio vulnificus* YJ016]	x	7E-67	95	NP_934531
	198	ET023977	336	8811	**RTX protein or Autotransporter adhesin [*Vibrio vulnificus* CMCP6]**	x	2E-072	42	NP_761533
	205	ET023978	450	894	glutamyl-tRNA synthetase [*Chromobacterium violaceum* ATCC 12472]	x	5E-31	50	NP_903103
	214	ET023979	355	5874	conserved hypothetical protein, putative DNA helicase [*Desulfovibrio vulgaris subsp. vulgaris* DP4]	x	7E-11	48	ZP_01458409
	216	ET023980	480	2034	**Chain A, Chondroitinase Ac Lyase [*Flavobacterium Heparinum*]**	x	7E-09	24	1CB8_A
	227	ET023981	414	498 732	GCN5-related N-acetyltransferase [*Psychromonas ingrahamii* 37] hypothetical protein P3TCK_08758 [*Photobacterium profundum* 3TCK]	x	7E-21 1E-05	57 89	ZP_01350703 P3TCK_08758
	260	ET023982	432	1071	transposase, IS4 [*Shewanella baltica* OS195]	x	8E-17	73	ZP_01432822
	269	ET023983	403	_	Unknown COG	x	_	_	_
	273	ET023984	367	801	ISPsy9, transposase OrfB [*alpha proteobacterium* HTCC2255]	x	2E-33	68	ZP_01448232
	278	ET023985	369	951	peptide ABC transporter, permease protein *[Brucella abortus* biovar 1 str. 9-941]	x	2E-24	45	YP_223694
	289	ET023986	449	1947	putative epimerase/dehydratase [*Vibrio parahaemolyticus* RIMD 2210633]	x	4E-77	96	NP_796614
	293	ET023987	447	_	Unknown COG	x	_	_	_
	318	ET023988	506	1194	putative ABC transporter [*Actinobacillus actinomycetemcomitans*]	x	3E-22	33	BAA82537
	320	ET023989	281	1080	Binding-protein-dependent transport systems inner membrane component [*Psychromonas ingrahamii* 37]	x	5E-14	62	ZP_01350492
	342	ET023990	383	510	hypothetical protein PBPRB0091 [*Photobacterium profundum* SS9]	x	3E-40	62	YP_131764
	348	ET023991	348	1530	deoxyguanosinetriphosphate triphosphohydrolase [*Photobacterium profundum* SS9]	x	7E-32	60	YP_130718
	351	ET023992	365	801	ISPsy9, transposase OrfB [*alpha proteobacterium* HTCC2255]	x	2E-20	61	ZP_01449847
	368	ET023993	517	1227	2-oxoisovalerate dehydrogenase alpha subunit [*Oceanicaulis alexandrii* HTCC2633]	x	6E-36	54	ZP_00953146
	376	ET023994	436	10947	alpha-aminoadipyl-L-cysteinyl-D-valine synthetase [*Amycolatopsis lactamdurans*]	x	6E-20	40	CAA40561
	384	ET023995	319	1164	nucleotide sugar dehydrogenase [*Vibrio vulnificus*]	x	6E-42	96	AAO32664
	417	ET023996	333	1143	hypothetical protein VP1567 [*Vibrio parahaemolyticus* RIMD 2210633]	x	2E-07	37	NP_797946
	424	ET023997	323	_	Unknown COG	x	_	_	_
	430	ET023998	372	_	Unknown COG	x	_	_	_
	431	ET023999	227	1155	putative acyl-CoA dehydrogenase [*Oceanospirillum sp.* MED92]	x	4E-25	72	ZP_01165327
	439	ET024000	421	1056	putative ATP-binding ABC transporter [*Rhizobium leguminosarum* bv. *viciae* 3841]	x	1E-30	47	CAK10505
	458	ET024001	252	8361	**mcyA [*Microcystis aeruginosa*]**	x	1E-14	54	BAA83992
	461	ET024002	284	2343	organic solvent tolerance protein [*Vibrio parahaemolyticus* RIMD 2210633]	x	9E-37	72	BAC58602
	476	ET024003	439	6348	putative non-ribosomal peptide synthetase [*Erwinia carotovora subsp. atroseptica* SCRI1043]	x	3E-22	44	YP_048600
	486	ET024004	481	1023	**capsule biosynthesis protein capA [*Bacteroides thetaiotaomicron* VPI-5482]**	x	4E-14	50	NP_810259
	490	ET024005	444	_	Unknown COG	x	_	_	_
	505	ET024006	468	2022	methyl-accepting chemotaxis protein [*Colwellia psychrerythraea* 34H]	x	3E-23	54	YP_270583
	507	ET024008	384	_	Unknown COG	x	_	_	_
	526	ET024009	482	1035	PTS system N-acetylgalactosamine-specific IID component [*Symbiobacterium thermophilum* IAM 14863]	x	3E-30	53	YP_075076
	541	ET024010	422	5835	unknown [*Pseudomonas syringae pv. syringae*]	x	8E-33	51	AAK83337
	553	ET024011	369	2157	probable toxin transporter [*Pseudomonas aeruginosa* PAO1]	x	4E-38	66	AAG07530
	563	ET024012	374	1434	Tn7-like transposition protein C [*Shewanella baltica* OS155]	x	3E-34	60	ZP_00584340
	566	ET024013	404	942	hypothetical protein OS145_02860 [*Idiomarina baltica* OS145]	x	4E-13	52	ZP_01041994
	595	ET024014	395	2145	type III restriction enzyme, res subunit [*Shewanella frigidimarina* NCIMB 400]	x	3E-32	65	YP_750771
	617	ET024015	191	_	Unknown COG	x	_	_	_
	618	ET024016	230	888	3-hydroxyisobutyrate dehydrogenase [*Marinomonas* sp. MED121]	x	6E-17	60	ZP_01075946
	622	ET024017	390	1124	hypothetical protein V12B01_04853 [*Vibrio splendidus* 12B01]	x	7E-32	79	ZP_00990364

Table 2. Summary of the sequence analysis of clones inserts specific for pathogenic strains of *V. nigripulchritudo* and absent from non pathogenic strains. Virulence gene candidates are indicated in bold characters.