Identification of a plasmid associated with virulence in *Vibrio nigripulchritudo*, a pathogen of the shrimp *Litopenaeus stylirostris*

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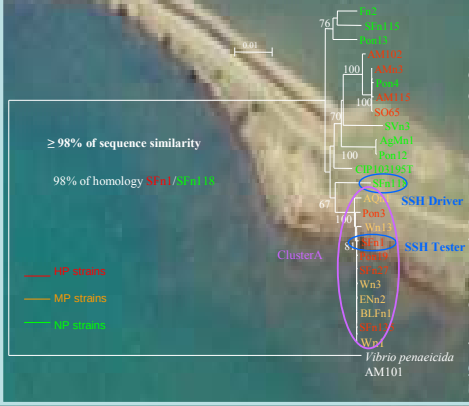
INTRODUCTION

Since 1997, a new pathology occurred seasonally in new caledonian shrimp farms during the warm season. Diseased *Litopenaeus stylirostris* shrimp suffered from a septicemic vibriosis which was attributed *to V. nigripulchritudo*. Until now, only three farms among 19 are affected by the so called "summer syndrome". Because the New Caledonian shrimp production is also affected by another vibriosis, namely syndrome 93 occuring in this case during the cool season (Goarant *et al.*, 2004), 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* strains have brought to light different virulence levels according to experimental infection (Goarant, 2005, 2006a); three virulence statuses were defined: highly pathogenic (HP), moderatly pathogenic (MP) and non pathogenic (NP). The aim of this work was to genetically characterize virulent *V. nigripulchritudo* strains. In a first step the genetic diversity of a collection of *V. nigripulchritudo* strains was analyze by MLST (MultiLocus Sequence Typing); in a second step, genetic markers of virulence were identified by a Suppressive Subtractive Hybridization performed between the genomes of a HP strain and a genetically close, NP isolate ; the distribution of the screened SSH fragments was studied in a selection of both virulent (either HP or MP) and NP *V. nigripulchritudo* strains 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 plasmid detected only in HP *V. nigripulchritudo* strains, leads to a discussion of the role of mobile genetic elements in the emergence of pathogenicity in *V. nigripulchritudo*.

Study of the genetic structure of *V. nigripulchritudo* using MLST

(Goarant *et al.*, 2006b)

MLST :
Reference genotyping method. A phylogenetic concatemeric tree based on the sequencing of six genes (3630 bp) was constructed by Neighbour Joining method and Kimura's 2 parameters distance.



Phylogenetic analysis:

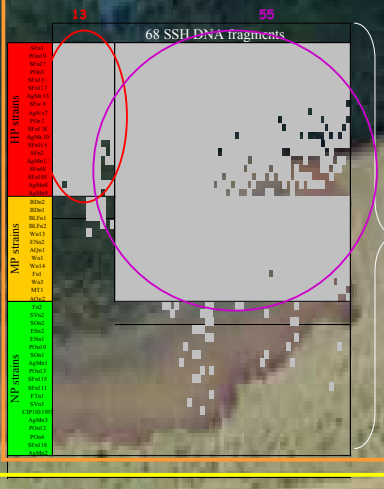
- Regarding genetic structure of the sub-selection of strains, one particular cluster with very low genetic variability groups only virulent strains for *L. stylirostris* (cluster A) and concern all HP strains isolated in a summer syndrome context
- The hypothesis of the emergence of this particular 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 MP strain to become HP
- Selection of the Tester SFn1 and the Driver SFn118 for the SSH approach according to their strong genetic identity and their opposite virulence statuses
- Any of those typing method allowed to differenciate HP strains from MP strains inside the Cluster A, that's why a molecular typing approach based on the detection of genetic markers of virulence appears necessary

SSH

A SSH DNA library was constructed between SFn1 and SFn118
Differential DNA fragments between SFn1 and SFn118 were screened by DNA-DNA hybridization and then sequenced
→ 1112 clones tested,
622 SSH DNA fragments specific of SFn1
521 DNA fragments sequenced

Macro-array

Distribution of the screened SSH fragments was studied in a selection of 52 HP and MP strains of the cluster A and NP strains, by DNA-DNA hybridization



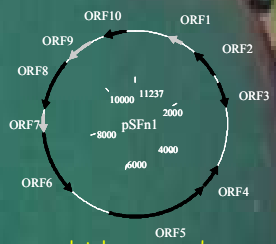
68 DNA fragments selected:
- 13 were found only in the HP strains o the cluster A associated to the summer syndrome
- 55 were present in both HP and MP strains of the cluster A

Putative genes involved in virulence process:
- cyanobacterial toxin mcyA
- Capsule
- Sidérophore
- RTX toxin

pSFn1, a plasmid associated with virulence of *V. nigripulchritudo* (Reynaud *et al.*, submitted)

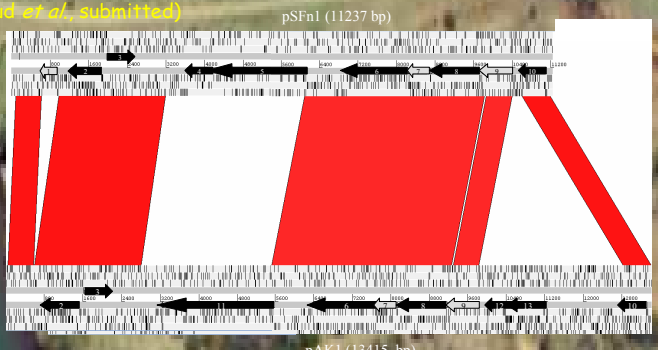
An identical plasmid was successfully extracted in all the HP strains of the cluster A (datas not shown): Identical restriction profils with EcoRI and XhoI were obtained.

The plasmid pSFn1 was completely sequenced
- 10 putatives ORFs were identified (graphic map of pSFn1)
- 10 of the specific DNA fragments of the HP strains of the cluster A identified by macro-array were localized on pSFn1
- among them, 3 present **strong identities** with sequences found in the 13,5kbp plasmid of *V. shilonii*, associated with coral bleaching events of *Oculina patagonica* (Rosenberg et Falkovitz, 2004)



pSFn1 could be considered as a plasmid linked to virulence necessary to increase the virulence in MP strains. no ORFs annotated in the plasmid can be assigned clearly to a virulence factor, nevertheless several hypotheses can be made concerning the role of pSFn1 genes in the virulence process: pSFn1 could either bring new virulence, or carry a regulator that would modulate the expression/function of pre-existing (possibly chromosomal) virulence factors

The plasmid pAK1 was completely sequenced
Syntheny analysis highlight that 5 regions were significantly similar between pSFn1 and pAK1 (score>500): 71.8% of pSFn1 was shared with pAK1 , with 93% of nucleotidic identity for these sequences. We can speculate that some strains of *V. nigripulchritudo* have recently acquired this plasmid or a part of this plasmid from *V. shilonii* or from another bacterial species in the environment via hoizontal gene transfert



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 red lines between the plasmid represent DNA:DNA similarities (BlastN matches between the two sequences, score>500).

Conclusion:

In next future, these results would permit the development of relevant diagnostic tools that would prove useful for accurate epidemiological survey of the summer syndrome. Among the pSFn1 ORFs identified and the fragments selected by macro-array, several could correspond to virulence gene candidates, however the cloning and sequencing of the entire gene followed by analyses of deletion mutants will be essential for the formal demonstration of the predicted, or supposed, role of a candidate gene: gene knock out strategy will be performed in order to break down the virulence mechanisms "gene by gene". Studying the distribution of this particular plasmid pSFn1 (or plasmid family) among the bacterial flora of the lagoon ecosystem could help to evaluate its relation to virulence for marine invertebrates and thereby contribute to a better prospective study of virulence gene circulation within this ecosystem.

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