VIBRIO2005

Program & Abstract book

November 6 - 8, 2005

Ghent, Belgium

http://lmg.ugent.be/vibrio2005

edited by Dirk Gevers and Peter Dawyndt

Preface

Dear colleagues,

A warm welcome to the first international conference on "The Biology of Vibrios" in Ghent. This two-day conference provides scholars from around the world with the opportunity to focus on priority themes in *Vibrio* research and to discuss the issues of future research.

There have been tremendous developments in the study of the biology of vibrios over the last two decades. Nearly 80 species are officially recognized today, some of which have well known ecological roles in nature. *V. cholerae* remains one of the main scourges of mankind, killing thousands of people yearly worldwide. Other vibrios e.g. *V. anguillarum*, *V. harveyi* and *V. salmonicida* are threats to reared marine animals. Vibrios are abundant in the marine environment and within the tissues/organs of several hosts e.g. fish and shellfish. More recently, certain vibrios have been associated with the mortality of corals and other cnidarians, worldwide. A range of biotechnological applications e.g. vaccine development, environmental monitoring and production of bioactive compounds are currently underway. Seven species have their whole-genome sequences available or approaching completion allowing detailed genomic and post-genomics analyses.

Clearly, it was timely to bring together researchers committed to the study of the biology of vibrios. The goal of this meeting is to have a forum of discussion of the present knowledge on vibrios as well as to identify the main research needs for future projects. Cutting-edge studies in Biodiversity, Ecology & Applications, Genomics, and Disease & Epidemiology will be presented during Vibrio 2005.

I wish you all a fruitful conference,

Prof. Dr. ir. Jean Swings
The Organizing Committee

VIBRIO2005 ORGANIZATION

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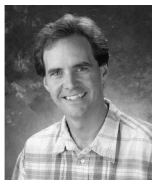

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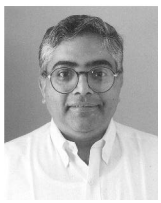

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Dr. Dirk Gevers
Ms. Annemie Struyvelt

Monday, November 7

8h-8h30: Registration / Poster preparation

Plenary Session 1: Biodiversity / Taxonomy
Chairs: Brian Austin and Fabiano Thompson

8h30 - 9h20: **Rita R. Colwell**, University of Maryland, USA
KEYNOTE: A Global Perspective for the Genus *Vibrio*

9h20 - 9h45: **Fabiano L. Thompson**, CBMAI, Brazil.
Current trends in the vibrios taxonomy

9h45 - 10h10: **Paul V. Dunlap**, University of Michigan, USA
Phylogenetic analysis of co-speciation in bioluminescent symbiosis

10h10 - 10h40: **Coffee break / Poster session**

10h40 - 11h30: **Martin Polz**, MIT, USA
KEYNOTE: Vibrio diversity in the Coastal Ocean: genomes, populations and species

11h30 - 11h55: **Carlos R. Osorio**, University of Santiago de Compostela, Spain
Identification of a subspecies-specific operon involved in siderophore biosynthesis and transport in *Photobacterium damselae* subsp. *piscicida*: evolutionary implications

11h55 - 12h20: **Bruno Gomez-Gill**, CIAD, Mexico
Collection of aquatic important microorganisms

12h20 - 12h30: Group photo

12h30 - 14h00: Lunch break / Poster session

Plenary Session 2: Ecology & Application

Chairs: Diane McDougald and John H. Paul, III

14h00 - 14h50: **Diane McDougald**, The University of New South Wales, Australia
KEYNOTE: Adaptive responses of vibrios

14h50 - 15h15: **Sirajul Islam**, ICDDR, Bangladesh
Blue-green algae as a long-term reservoir of *Vibrio cholerae* O1 in the aquatic environment of Bangladesh

15h15 - 15h40: **Daniel P Keymer**, Stanford University, USA
Linking ecological niche and diversity of *Vibrio cholerae* using complete genome hybridization and phenotypic comparisons

15h40 - 16h10: **Coffee break / Poster session**

16h10 - 17h00: **John H. Paul, III**, University of South Florida, USA
KEYNOTE: The ecology and genomics of two marine vibriophages

17h00 - 17h25: **Guillaume de Magny**, IRD, France
Regional-scale synchrony of cholera epidemics in the Equatorial Atlantic Coast (gulf of Guinea)

17h25 - 17h50: **James D. Oliver**, University of North Carolina, USA
Recent findings on the *Vibrio vulnificus* genome, and on in situ gene expression

20h00 - 23h00: Conference dinner

Tuesday, November 8

Plenary Session 3: Genomics

Chairs: Tetsuya Iida and John F. Heidelberg

8h30 - 9h20: **John F. Heidelberg**, TIGR, USA
KEYNOTE: Comparative Genomics of *Vibrio cholerea*

9h20 - 9h45: **Amy L. Schaefer**, University of Wisconsin, USA
Coming out of the dark: using genomics to shed light on the squid-vibrio symbiosis

9h45 - 10h10: **Tetsuya Iida**, Osaka University, Japan
Pathogenic mechanism and life cycle of a marine bacterium *Vibrio parahaemolyticus*: an insight from genome sequence

10h10 - 10h40: Coffee break / Poster session

10h40 - 11h30: **Yan Boucher**, Macqurie University, Australia
KEYNOTE: The role of LGT in Vibrio evolution: dynamics of chromosomal integrons

11h30 - 11h55: **Frédérique Le Roux**, Ifremer-Institut Pasteur, France
Complete genome sequence of *Vibrio splendidus*: a pathogenic bacterium for a wide range of marine animals

11h55 - 12h20: **David W. Ussery**, CBS, Denmark
Bioinformatic Tools to Compare Vibrio Genomes

12h20 - 14h30: Lunch break ("Het Groot Vleeshuis", at walking distance)

Plenary Session 4: Disease & Epidemiology

Chairs: Karl Klose, Balakrish Nair and Frans Ollevier

14h30 - 15h20: **Shah M. Faruque**, ICDDR, Bangladesh
KEYNOTE: Current concepts in cholera dynamics

15h20 - 15h45: **Tom Defoirdt**, Ghent University, Belgium
Disruption of quorum sensing protects gnotobiotic artemia from pathogenic *Vibrio harveyi* and*Vibrio campbellii*

15h45 - 16h10: **Jorge H. Crosa**, Oregon Health & Science University, USA
Iron transport and virulence of the fish pathogen *Vibrio anguillarum*

16h10 - 16h40: Coffee break / Poster session

16h40 - 17h05: **Karl Klose**, University of Texas, USA
KEYNOTE: Effect of flagellar synthesis on *Vibrio cholerae* virulence and biofilm formation

17h05 - 17h30: **Joachim Reidl**, Universitet Wurzburg, Germany
Vibrio cholerae and lipopolysaccharide: analysis of the O antigen transfer

17h30 - 17h55: **Yannick Labreuche**, Ifremer, France
An overview on the pathogenesis of *Vibrio aestuarianus* strain 01/32 to the pacific oyster *Crassostrea gigas*

17h55 - 18h20: **David R. Nelson**, University of Rhode Island, USA
Identification and characterization of two hemolysin gene clusters in *Vibrio anguillarum*

18h20 - 18h45: Conclusions on future priorities in *Vibrio* research

19h00 - 21h30: Dinner: Tasting of regional products

- Poster Index -

Biodiversity & Taxonomy

B-01 **Rosana Ferreira** : Signaling by a GGDEF/ EAL – containing protein mediates regulation of gene expression in *Vibrio parahaemolyticus*

B-02 **Swapan Banerjee** : Genotypic and Phenotypic Characterization of *Vibrio parahaemolyticus* isolated from Molluscs at Harvest and from Clinical Sources in Canada.

B-03 **Teresa P. Nieto** : Diversity of *Vibrio* species associated with the cultured Galician marine organisms

B-04 **Nina I. Smirnova** : Biodiversity of *Vibrio cholerae* Strains Isolated in Russia: Genomic Structure and Molecular Typing

B-05 **Edward Feil** : Characterisation of a natural population of *Vibrio* sp. using MLSA

B-06 **Kumiko Kita-Tsukamoto** : Rapid identification of marine luminescent bacteria by amplified ribosomal DNA restriction analysis (ARDRA)

B-07 **Regine Vieira** : Impact of the shrimp farming activity on *Vibrio* spp. diversity in Ceará State's estuaries

B-08 **Ana Carolina Vicente** : Occurrence and Composition of Class 1 and Class 2 Integrons in Clinical and Environmental O1 and Non-O1/Non-O139 *Vibrio cholerae* strains from the Brazilian Amazon Region

B-09 **Regine Vieira** : Phenotypic resemblance among *Vibrio alginolyticus* strains isolated in north-western Adriatic Sea and in south-western Atlantic Ocean

B-10 **Cynthia T. Hedreyda** : Partial *gyrB*, *toxR*, hemolysin and *lux* Gene Sequences As Alternative Markers to Differentiate *Vibrio harveyi* from *Vibrio campbellii*

B-11 **Roxana Beaz** : *Vibrio* species associated with the culture of clams in the north-west of Spain.

B-12 **Susana Prado** : New vibrios isolated from disease outbreaks in shellfish larvae

B-13 **Fitri Fegatella** : Molecular characterization of *Vibrio* isolated from shrimp hatcheries in Indonesia

Ecology & Application

E-01 **Frank K. Thomson** : Ships' ballast as a potential vector for the transfer of antibiotic-resistance genes of *Vibrio cholerae*

E-02 **Munirul Alam** : *Vibrio cholerae* O1 and O139 in the Aquatic Environment of Mathbaria, Bangladesh

E-03 **Ji-Dong Gu** : Characterization of cryptic plasmids from environmental *Vibrio* species isolated from coastal marine environments

E-04 **Rohinee Paranjpye** : Role of *Vibrio vulnificus* type IV pili in persistence in oysters, *Crassostrea virginica.*

E-05 **Else M Fykse** : Sensitive and rapid detection of viable *Vibrio cholerae* cells by a Nucleic Acid Sequence Based Amplification (NASBA) method in water

E-06 **Jaran S. Olsen** : Development of a MLVA analysis for genetic fingerprinting of *Vibrio cholerae*

E-07 **Tomoo Sawabe** : Construction of a set of green fluorescent protein-tagged vibrios for studying host-microbe interactions

E-08 **Henning Sorum** : Adaptive features in cold water *Vibrio* pathogens

E-09 **Machi Sato** : TCA cycle components as the resuscitation factors of *Vibrio parahaemolyticus* from VBNC state

E-10 **S. N. Venter** : Population dynamics of *V. cholerae* in an inland catchment in South Africa

E-11 **Jennifer Hsieh** : Environmental Factors Influencing *Vibrio* sp. Population Dynamics

E-12 **Mitsuru Eguchi** : Survival of Vibrio anguillarum, a fish pathogen, in freshwater by forming biofilms

E-13 **Mitsuru Eguchi** : Cloning and transcriptional regulation of Na+-NQR gene of *Vibrio anguillarum*

E-14 **Marius Vital** : Vibrio cholerae O1 grows well with natural assimilable organic carbon (AOC) in different types of freshwater

Genomics

G-01 **Luis Caetano M. Antunes** : Analysis of *lux* Box Nucleotides Required for Activity of the *Vibrio fischeri* LuxR Protein

G-02 **Steinar M. Paulsen** : Proteome analysis of the fish pathogen *Vibrio salmonicida* by 2-D gel electrophoresis and mass spectrometry

G-03 **Michael C. Miller** : Natural Competence and Variation in *Vibrio cholerae* Genomes: Implications for Function and Evolution

G-04 **Anne-Marie Quirke** : High genome plasticity within *V. vulnificus* is revealed by molecular analysis of genomic islands among 28 isolates.

G-05 **Catherine Hurley** : identification of genomic islands unique to pandemic *V. parahaemolyticus* O3:K6 isolates

G-06 **Johan BINESSE** : Suppression Subtractive Hybridization (SSH) to explore strains specific genes of *Vibrio splendidus* related strains

G-07 **Shinji YAMASAKI** : Genetic Diversity of Super-Integron in various *Vibrio cholerae* strains

G-08 **Salvador Almagro Moreno** : Molecular evolutionary analysis of the sialic acid metabolism gene cluster (*nan-nag*) among bacteria

G-09 **Gaël Erauso** : Comparative genomics of two large plasmids present in the marine mollusc pathogen *Vibrio tapetis* type strain CECT4600

G-10 **Cédric Lemaire** : Detection of *Vibrio cholerae* in seafood using real time PCR

G-11 **Tim T. Binnewies** : Comparison of *Vibrio* genomes

Disease & Epidemiology

D-01 **Karen L. Visick** : The symbiosis polysaccharide (*syp*) gene cluster promotes symbiotic colonization and biofilm formation by *Vibrio fischeri*

D-02 **C. B. Munn** : Properties of vibrios associated with tropical Pacific corals

D-03 **J. Pike** : Association of vibrios with tissue necrosis in the pink sea fan *Eunicella verrucosa*

D-04 **Ivan A. Dyatlov** : Development and production of preparations for cholera prophylaxis and diagnosis in the Russian Federation

D-05 **Tatsuya Nakayama** : Recognition of various toxins from *Vibrio carchariae* and *Vibrio harveyi*

D-06 **Frans Ollevier** : Impact of *Vibrio proteolyticus* on probiont uptake by *Artemia franciscana*

D-07 **Aklak Miah** : Molecular Typing: Randomly amplified polymorphic DNA (RAPD) studies to evaluate discriminatory abilities between virulent and avirulent isolates of *Vibrio parahaemolyticus*

D-08 **Matthieu Garnier** : Phylogenetic diversity and ecology of *Vibrio aestuarianus*, a pathogen of oysters *Crassostrea gigas*

D-09 **Dominique Hervio-Heath** : Real time PCR for sensitive detection of total and pathogenic *Vibrio parahaemolyticus* in estuary water in Southern Brittany, France.

D-10 **Yann Reynaud** : Searching for genetic markers of virulence in *Vibrio nigripulchritudo*, a *Litopenaeus stylirostris* shrimp pathogen isolated in New-Caledonia

D-11 **Sariqa Wagley** : Detection and Characterisation of Total and Haemolysin-producing *Vibrio parahaemolyticus* in Bivalve Molluscan Shellfish from U.K Growing Waters

D-12 **Tomoo Sawabe** : Mass mortality of Japanese abalone *Haliotis discus hannai* caused by *Vibrio harveyi* infection

D-13 **Laura Masini** : Characterization of virulence factors in *V. cholerae* non-O1, non-O139 environmental and clinical isolates

D-14 **F. Jerry Reen** : Functional analyses of the toxin-coregulated pilus protein TcpA from toxigenic *V. cholerae* isolates.

D-15 **S. N. Venter** : Cholera outbreak in KwaZulu-Natal, South Africa: 2000 - 2004

D-16 **Bernard China** : Detection and characterization of *Vibrio parahaemolyticus* in seafood using real time PCR.

D-17 **G. B. Nair** : Molecular analysis and significance of El Tor strains of *Vibrio cholerae* O1 that carry some genes of the classical biotype

D-18 **Paillard Christine** : Comparative phenotypic and genetic characteristics and virulence properties of *Vibrio tapetis* and related strains isolated from molluscs and fishes suffering vibriosis

D-19 **Marie-Laure Quilici** : Non-cholera Vibrios infections in France, 1995-2004: ten years of surveillance

D-20 **Annick Robert-Pillot** : Detection and quantification of *V. cholerae* and *V. parahaemolyticus* in pure cultures, oysters and water samples by real-time PCR

D-21 **Marie-Agnès Travers** : *Vibrio carchariae* detection and interaction with *Haliotis tuberculata* haemocytes

D-22 **Jean-Michel Fournier** : Crystal structure of a monoclonal antibody directed against an antigenic determinant common to Ogawa and Inaba serotypes of *Vibrio cholerae* O1

D-23 **Carmen Amaro** : New strategies for the isolation and identification of the eel and human pathogen *Vibrio vulnificus* serovar E

D-24 **Duncan Colquhoun** : Characterisation of Norwegian *Vibrio anguillarum* serotype O2 by pulsed-field gel electrophoresis

D-25 **Meiying Yan** : The live cholera vaccine candidates IEM108 and IEM109 can elicit protective immunity and resistance to CTXF infection in animals

D-26 **Colin B. Munn** : Effect of temperature on long term survival and induction of the viable but non-culturable state in the coral-associated bacteria *Vibrio shiloi* and *Vibrio tasmaniensis*

- Speaker abstracts -

- Plenary session 1 -

Biodiversity & Taxonomy

A Global Perspective for the Genus *Vibrio*

Rita R. Colwell

University of Maryland and Johns Hopkins University

The genus *Vibrio* traces back to the earliest days of bacteriology, at the turn of the twentieth century. Now, over a hundred years later, the genus has expanded to include species that play significant roles in the natural cycles of life and in the health of the planet, both human and animal, in the latter case significantly so for fish and shellfish of the world oceans. It is now possible to monitor from space the conditions conducive to growth and proliferation of vibrios, notably *Vibrio cholerae*, that are linked to human pandemics. Furthermore, the ancestral strains of *Vibrio* can be tracked to the deepest regions of the world oceans, lending further credence to the global role of vibrios in life on the blue planet.

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Current trends in the vibrios taxonomy

F. L. Thompson[1], D. Gevers[2], C. C. Thompson[1], P. Dawyndt[2], B. Hoste[2], & J. Swings[2]

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The taxonomy of vibrios has been under extensive scrutiny in the last years leading to new genera and several new species descriptions, reclassifications and the development of improved identification tools. The nearly 80 species currently recognized show different degrees of genomic heterogeneity and geographical distribution. To date, accurate identification of vibrios

at the family and genus level is obtained by 16S rRNA gene sequencing, whereas identification at species and strain levels require the application of genomic analyses, including DNA-DNA hybridisation, rep-PCR and AFLP. Because several vibrios have nearly identical 16S rRNA sequences and similar phenotypic features, genomic fingerprinting has become an essential tool for reliable species and strain identification. Because inter-laboratory comparisons of genomic fingerprint patterns are difficult and restricted to a few reference laboratories, the sequencing of house-keeping genes has emerged as an alternative (Gevers et al., 2005).
We have analysed the usefulness of *rpo*A, *rec*A, *pyr*H, *uvr*B, *atp*A, and *obg* gene sequences for the identification of vibrios (Thompson et al., 2005). Fragments of these loci were sequenced from a well-documented *Vibrionaceae* type and reference strain collection. The phylogenetic trees constructed with the different genetic loci were roughly in agreement with former polyphasic taxonomic studies, but some pairs of related species e.g. *V. coralliilyticus* and *V. neptunius* changed position in the different trees. The families *Vibrionaceae*, *Photobacteriaceae*, *Enterovibrionaceae*, and *Salinivibrionaceae* were all differentiated on the basis of each genetic locus. The genus *Vibrio* was heterogeneous and polyphyletic, with *V. fischeri*, *V. logei* and *V. wodanis* grouping closer to *Photobacterium*. *V. halioticoli*-, *V. harveyi*-, *V. splendidus*-, and *V. tubiashii*-related species formed groups within the genus *Vibrio*. Each species clearly formed separated clusters with at least 98, 94, 94 % *rpo*A, *rec*A and *pyr*H gene sequence similarity respectively. We will present an overview of the main polyphasic taxonomic findings of the last years and discuss the impact of the genomic electronic automatic taxonomy in vibrios.

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, Van de Peer Y, Vandamme P, Thompson FL, Swings J. Re-evaluating prokaryotic species. Nat Rev Microbiol. 2005 Sep;3(9):733-9.

Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. Appl Environ Microbiol. 2005 Sep;71(9):5107-15.

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Phylogenetic analysis of co-speciation in bioluminescent symbiosis

Paul V. Dunlap[1,*], Jennifer C. Ast[1], Seishi Kimura[2], and Atsushi Fukui[3]

[1]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan, USA; [2]Fisheries Research Laboratory, Mie University, Shima, Mie, Japan; [3]School of Fisheries and Marine Technology, Tokai University, Shimizu-Orido, Shizuoka, Japan.

Bioluminescent symbiosis between luminous bacteria and marine squids and fishes might provide a compelling example of symbiont-host co-speciation, with implications for co-evolutionary theory. These associations, which persist over the lifetime of the host, are highly specific; members of a given family of host animal typically harbor a single, often different, species of luminous bacteria. To house the bacteria, the animal elaborates a complex light organ, the

tissues of which also serve to control and direct the bacterial light, which the animal uses in various luminescence displays associated with mating, avoiding predators and attracting prey. Furthermore, in some cases it has not yet been possible to culture the bacteria independent of the animal, suggesting that the bacteria are obligate symbionts. These attributes apparently provide opportunities for and might reflect co-speciation selection. Alternatively, co-speciation might not characterize bioluminescent symbiosis. The bacteria in many cases are facultative symbionts that occur also in habitats other than the host light organ and are known or thought to be acquired from the environment by the members of each new host generation. These attributes indicate that the bacteria are likely to be subject to selection pressures not consistent with an obligate dependence on the animal. This ambiguity led us to directly test the co-speciation hypothesis, using deep-sea fishes, the most taxonomically diverse of the symbiotically bioluminescent hosts, as a suitable test case. Specimens representing 16 species in five families from four teleost orders, Chlorophthalmidae (Aulopiformes), Macrouridae and Moridae (Gadiformes), Trachichthyidae (Beryciformes), and Acropomatidae (Perciformes), were sampled. The fish phylogeny was reconstructed using mitochondrial 16S rRNA and cytochrome oxidase I gene sequences. Luminous bacteria were isolated from the light organs of these fishes. To ensure as rigorous a test of co-speciation as possible, the bacterial strains were taken from the light organs of the specimens used to reconstruct the fish phylogeny, and multiple strains were analyzed. The bacterial phylogeny was reconstructed using the *recA*, *gyrB* and *luxA* genes. Parsimony-based phylogenetic analysis revealed deep divergences for the fishes, as predicted by their taxonomy; the phylogenetic divergence of each order, family and species of fish was distinct and robust. For the bacteria, initial studies had indicated that strains from some of these fishes represented a new species, "*Photobacterium kishitanii*". That finding was confirmed here and extended to each of the fishes examined. Regardless of the fish species, family or order from which the symbiotic light-organ bacteria were isolated, the bacteria were found to represent a single species, "*P. kishitanii*". Furthermore, the bacterial strains formed a tightly clustered clade and exhibited little within-species diversity; essentially identical strains were isolated from fishes in different families and orders. These results establish that a single, well-resolved and tightly clustered clade of luminous bacteria serves as symbiont to a phylogenetically diverse array of fishes. Consistent with these findings, direct comparison revealed no topological congruence between the fish and bacterial phylogenies; evolutionary divergence of the host fishes was not matched by a similar pattern of diversification in their symbiotic bacteria. These results demonstrate that host-symbiont co-speciation does not characterize bioluminescent symbiosis.

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Vibrio diversity in the Coastal Ocean: genomes, populations and species

Martin Polz[1]

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Vibrios are long recognized members of oceanic bacterioplankton yet the diversity and dynamics in their natural environment remains poorly explored. At a coastal ocean site, we regularly detect 19 co-existing *Vibrio* (and closely related *Photobacterium*) taxa. Members of these are organized into phylogenetically distinct clusters, which display distinct and recurrent patterns of occurrence over annual cycles. When examined in detail, one such cluster (related to *Vibrio splendidus*) contained over a thousand well differentiated (in size and gene sequences) genome types, each occurring on average at such low environmental concentration that unique traits among them must have negligible impact on overall population function. Indeed, these strains display remarkable diversity in ability to grow on distinct carbon substrates despite isolation at a single site, so it is difficult to imagine that each arose in adaptation to patterns of co-occurrence of carbon substrate availability in the plankton. The question therefore arises how these strains are partitioned into populations at the functional and evolutionary level. On the other hand, the high genetic, genomic and functional diversity among bacterioplankton we have recently observed suggests that ecologically distinct populations may contain only 10^3 to 10^6 individuals and that on the order of one Mole (~10^{23}) of differentiated prokaryotic populations exist in the Ocean. These possibilities are discussed in the context of mechanisms of genome evolution and differentiation.

Thompson, J.R., Randa, M.A., Marcelino, L..A., Tomita, A., Lim, E., Polz, M.F. (2004) Diversity and dynamics of a North Atlantic coastal vibrio community. Appl. Environ. Microbiol. **70(7):**4103-4110.

Acinas, S.G., Klepac-Ceraj, V., Hunt, D.E., Pharino, C., Ceraj, I., Distel, D.L., Polz, M.F. (2004) Fine-scale phylogenetic architecture of a complex bacterial community. Nature. **430:**551-554.

Randa, M.A., Polz, M.F., Lim, E. (2004) Population dynamics of Vibrio vulnifucus in a North Atlantic estuary. Appl. Environ. Microbiol. **70(9):**5469-5476.

Thompson, J.R., Pacocha, S., Pharino, C., Klepac-Ceraj, V., Hunt, D.E., Benoit, J., Sarma-Rupavtarm, R., Distel, D.L., Polz, M.F. (2005) Genotypic diversity within a natural coastal bacterioplankton community. Science. **307:**1311-1313.

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Identification of a subspecies-specific operon involved in siderophore biosynthesis and transport in *Photobacterium damselae* subsp. *piscicida*: evolutionary implications

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Photobacterium damselae is a marine gram-negative bacterium of the family *Vibrionaceae*, that includes strains classified into two distinct subspecies, namely subsp. *piscicida* and subsp. *damselae*. *P. damselae* subsp. *piscicida* is the causative agent of pasteurellosis, a disease affecting a number of fish species in marine aquaculture systems worldwide. *P. damselae* subsp. *damselae* affects a variety of marine animals and has also been isolated from fatal cases in humans. The taxonomical placement of the two subspecies within this species epithet has been controversial, since they show important phenotypical differences. Actually, until 1995 these subspecies were classified into different genera and families, as *Pasteurella piscicida* (family *Pasteurellaceae*), and *Vibrio damsela* (family *Vibrionaceae*) respectively. However, the two species show more than a 80% relatedness in their genomes, as evaluated by DNA-DNA hybridization, and their 16S gene sequences are 100% identical. The two subspecies have been reported to produce a different kind of siderophore, but the genetic basis of their iron-sequestering systems remains unknown. In the present study, we analyzed the pattern of proteins which are induced under conditions of low iron availability in both subsp. *piscicida* and subsp. *damselae* strains. Two high-molecular weight proteins were found to be induced under iron limitation in subsp. *piscicida* but not in subsp. *damselae*. Thereafter, suppression subtractive hybridization was carried out in order to survey for genes which are specific of *P. damselae* subsp. *piscicida*. As a result, several subsp. *piscicida*-specific clones were isolated and analyzed. Two clones were found to encode partial ORFs homologous to described non-ribosomal peptide synthetases involved in siderophore biosynthesis. Chromosome walking was employed to clone the genomic regions in the vicinity of the isolated fragments. A DNA region of ca. 22 Kb was entirely sequenced and characterized, and five open reading frames were inferred, which constituted an iron-regulated transcriptional unit, as demonstrated by RT-PCR assays. The operon included genes coding for a putative AraC-family transcriptional activator, a putative outer membrane ferrisiderophore receptor, a protein of unknown function, and two putative high-molecular weight non-ribosomal peptide synthesases (*irp2* and *irp1*) whose molecular weight was coincident with those subsp. *piscicida*-specific iron-induced protein bands mentioned above. Insertional inactivation of the *irp1* gene rendered *P. damselae* subsp. *piscicida* impaired for growth under iron limiting conditions, and unable to produce siderophores on a CAS agar plate assay. Studies on the presence of these genes in a collection of *P. damselae* strains demonstrated that this iron-regulated operon is specific of some subsp. *piscicida* isolates, being absent in a collection of ten subsp. *damselae* strains tested. Surprisingly, the amino acid

sequences of the proteins of this operon showed a high similarity to proteins involved in the production and utilization of siderophore yersiniabactin described in *Yersinia* species, while showing very low similarity to *Vibrio* proteins. This is in contrast with the high overall genetic similarity between described *P. damselae* genes and genes of *Vibrio* species. In the other side, we have also encountered in subsp. *damselae* strains a gene coding for a putative ferrisiderophore receptor which is highly similar to that of *V. parahaemolyticus*. When surveying this gene in subsp. *piscicida* strains it was found that an homologue exists, but the ORF is interrupted constituting a pseudogene. This supports previous observations reporting that these two subspecies produce and utilize two different kinds of siderophores. All these data suggest that, originally, the two *P. damselae* subspecies shared a common siderophore utilization system which is similar to those described in *Vibrio* species, whereas the genetic system currently encountered in subsp. *piscicida* strains could have been gained by lateral transfer, eventually substituting the function of the primitive *Vibrio*-like siderophore-mediated iron acquisition system. The occurrence of mutations leading to pseudogene formation, and the fact that this iron uptake system is crucial for multiplication into the host, could have lead to selective pressure favouring the acquisition of a new genetic system in subsp. *piscicida* by horizontal transfer. An *in silico* analysis of the domains present in the modular organization of the Irp1 and Irp2 proteins suggests that the siderophore produced by *P. damselae* subsp. *piscicida* is structurally related to yersiniabactin.

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Collection of aquatic important microorganisms

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The Collection of Aquatic Important Microorganisms (CAIM) is a collection dedicated to the study and preservation of bacterial strains obtained from aquatic systems and aquacultural facilities, which have mayor importance for the industry or the environment. Because the genus Vibrio is of primary relevance in the aquaculture industry as a pathogen and also a very common member of all marine-estuarine environments, the Collection is strongly focused to its study.

CAIM was formerly named Collection of Aquacultural Important Microorganisms, but changed to the actual name because the interests of the collection have now broaden also to the study microorganisms that have the aquatic environment as their habitat, and not only those restricted to aquaculture.

The Collection started as a research collection during 1995 and has evolved to be a service collection with limited service capabilities due to economic restrains. It is now registered at the World Federation for Culture Collections (WFCC) and has been gaining recognition among the researchers interested in the vibrios. It is housed in the Center for Research on Nutrition and Development (CIAD) at the Mazatlan Unit for Aquaculture and Environmental, Management. CIAD is a non-profit federal funded research centre in the state of Sinaloa, Mexico.

The Collection is now composed of 1790 strains cryopreserved at -70 °C and is currently making and effort to lyophilize all the strains. It has 73 type strains of the Vibrionaceae family and nearly 350 reference strains. It has been focused on collecting vibrios from penaeid shrimp, marine fish (snapper and puffer fish), oyster, and seawater. The collection has strains isolated from 29 different countries, but mainly from Mexico, Norway, Brazil, USA, and Belgium.76.7 % of the strains were acquired after 2000, and 19.4 % during 1990 and 1999. Until 2005, only 35 % of the strains were identified to species level, but thanks to a project by the Mexican government (SEMARNAT) the majority of the strains will be characterize molecularly by rep-PCR and identified.
Quality controls are being implemented, we now relay heavily on molecular methods as well as simple bacteriological techniques.
Strain information of CAIM will be incorporated to the Mexican National System on Information about Biodiversity thanks to a grant by CONABIO. This information will also be available through the World Information Network on Biodiversity.
The services offered by CAIM are preservation and distribution of strains, characterization and identification of strains by molecular methods (rep-PCR and 16S rRNA sequencing). Because we strongly believe in the free access to information and to non-modified biological material, the distribution of strains is free for the academia but has a fee for the industry or for-profit institutions. We also do not believe in the patenting of nature, therefore we do not accept "patented" bacteria in the collection. In order to continue to honor these commitments, we have to restrain the number of strains to be deposited and also the ones to be distributed. Strains are send via normal post in agar slants and a charge is applied to cover freight costs.

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- Plenary session 2 -

Ecology & Application

Adaptive Responses of Vibrios

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Survival of marine bacteria depends on efficient adaptation of cells to changes in environmental conditions. Members of the genus *Vibrio* are able to successfully proliferate in areas of high substrate availability and cell density (e.g. biofilms), as well as to persist as free-living pelagic cells. Vibrios have been found associated with higher organisms and with inanimate surfaces and can constitute a significant fraction of the gastrointestinal tract of fish and invertebrates. These associations may be symbiotic as is the case for *Vibrio fischeri* and the Hawaiian squid, or pathogenic as in the case of *Vibrio harveyi* which causes high mortality rates in prawns, especially in aquaculture ponds. To accommodate the shift from free-living to host-associated growth, the life cycles of individual cells may be complex. For example, under these conditions they will be faced with intervals of non-growth when free-living, followed by increased nutrient availability when host-associated along with new stresses such as host defenses. The ability to survive under these changing conditions indicates that vibrios have a diversity of adaptive responses. We have studied several aspects of adaptation that have been suggested to play a role in survival of vibrios. Starvation adaptation is important for coping with changing nutrient levels and also has been shown to induce cross-protection against other stresses, such as oxidative stress. Starvation also leads to a delay in the viable but non-culturable response, indicating that changes in central metabolism are important for regulating environmental adaptation. Biofilm formation, another type of developmental adaptation, is likely to play a role in the life cycle of Vibrios in the environment. One example of the role of biofilm formation is the protection from grazing pressure by protozoans. We have performed biofilm experiments using smooth and rugose variants of *Vibrio cholerae* and have observed that grazing pressure drives an increase in biofilm biomass and a switch

from the smooth 'explorer' strategy to the rugose 'persister' strategy. As mentioned above, cells need to be able to regulate adaptive phenotypes in response to changing environmental condition. Quorum sensing has been shown to regulate many of these responses in Vibrios, including starvation adaptation and oxidative stress responses. Furthermore, we will present data that links quorum sensing, to biofilm formation and resistance to grazing by protozoans.

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Blue-green algae as a long-term reservoir of *Vibrio cholerae* O1 in the aquatic environment of Bangladesh

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Cholera is endemic in Bangladesh and maintains a regular seasonal pattern. Cholera epidemics occur twice every year, the highest peak during post-monsoon season in winter and a second smaller peak during pre-monsoon season in summer. During cholera seasons, the causative agent *Vibrio cholerae* O1 could be isolated from the environment and patients but disappears from the environment during interepidemic periods. The interepidemic reservoir or sites of survival and multiplication of *V. cholerae* O1 were unknown until recently. It was also not known how the seasonality and endemicity of cholera in Bangladesh are maintained? Scientists have been trying to answer these questions for about hundred years. It was hypothesised that *V. cholerae* is an autochthonous flora of brackish and estuarine environment. As cholera is water borne disease, it was thought that the reservoir of cholera would be the aquatic fauna. Therefore, investigations were carried out to see the role of various aquatic fauna as possible reservoir of *V. cholerae* O1. Oyster, crab, snail, fish etc. have been investigated as possible reservoirs but there was no conclusive evidence that any of these aquatic fauna can act as a reservoir of *V. cholerae* O1. *V. cholerae* O1 secrets an enzyme chitinase and it was thought that *V. cholerae* O1 can be associated with chitinous zooplankton in the aquatic environment. A microcosm study showed that *V. cholerae* O1 can survive in association with copepod, a zooplankton at 0.2% salinity up to 14 days but in control water without copepod, *V. cholerae* O1 survived up to 4 days. *V. cholerae* O1 gets survival advantage in association with copepod than water without copepod. *V. cholerae* O1 also secrets an enzyme mucinase and the role of mucinase is to degrade mucin from the nature. The mucin is present in the cell wall of aquatic plants. Therefore it was also thought that *V. cholerae* O1 could also make an association with the mucin containing aquatic plants. Investigations were therefore conducted to see the role of aquatic plants as reservoir of *V. cholerae* in the aquatic environment of Bangladesh. Microcosms studies showed that *V. cholerae* O1 could survive longer (27 days) in association with *Ulva lactuca,* a marine alga and common duckweed, *Lemna minor* than the water on which the *Ulva lactuca* and *L. minor* were floating. However, the 27 days were not considered long enough to cover the interepidemic periods. It needs minimum 6 months to cover interepidemic periods. Therefore investigation continued and finally it was found that *V. cholerae*

O1 can enter inside the mucilaginous sheath of a blue-green alga, *Anabaena variabilis* and can survive for years. *V. cholerae* O1 remain viable but become non-culturable inside the mucilaginous sheath of *Anabaena variabilis*. It has been observed by phase contrast and immuno scanning electron microscopes that *V. cholerae* O1 can multiply and maintain their progeny generations after generations and survive years after years inside the mucilaginous sheath of *A. variabilis*. The viable but non-culturable *V. cholerae* O1 was detected using fluorescent antibody technique. It was also observed that *V. cholerae* O1 remains at different depths inside the mucilaginous sheath of *A. variabilis* that has been detected by confocal microscope. PCR and Southern blot hybridisation were carried out to detect the lower number of *V. cholerae* O1 from *A. variabilis*. Studies also showed that *Hapalosiphon* sp. and *Nostoc* sp. of blue-green algae can also act as long-term reservoirs of *V. cholerae* O1. It was observed that the haemagglutinin protease (*hap*) gene is involved for association between mucilaginous blue-green algae and *V. cholerae* O1. Chemotaxis experiments with wild type *hap*+ *V. cholerae* O1 and its isogenic mutant (*hap*-) showed that chemotaxis plays an important role to make the association between *V. cholerae* O1 and blue-green algae. One important functional aspect of blue-green algae and *V. cholerae* O1 is the exchange of carbon dioxide and oxygen. In the association of A. *variabilis* and *V. cholerae* O1, the algal host provides oxygen during photosynthesis that can be utilized in aerobic respiration by *V. cholerae*, which produces carbon dioxide, which may in turn be available for algal photosynthesis. Thus, *A. variabilis* and *V. cholerae* O1 may maintain a symbiotic relationship. The laboratory based and field based studies indicated that blue-green algae can act as a long-term reservoir of *V. cholerae* O1 in the aquatic environment of Bangladesh. The cholera seasons in Bangladesh coincided with the algal bloom formation in the aquatic environment of Bangladesh, which might explain the seasonal occurrence of cholera in Bangladesh. This study therefore provides some possible explanation of how the endemicity and seasonality of cholera in Bangladesh are maintained.

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Linking Ecological Niche and Diversity of *V. cholerae* using Complete Genome Hybridization and Phenotypic Comparisons

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Prediction of pathogen incidence and infectious disease outbreaks has long been sought after by public health administrators and environmental scientists. Recently, many investigators have attempted to link the presence of pathogenic microorganisms in the environment to changes in weather and climate. Outbreaks of cholera in Bangladesh, caused by the autochthonous marine bacterium *Vibrio cholerae*, have been shown to follow a seasonal pattern with increased cases of infection immediately following the monsoon season [Lipp *et al.*, 2002]. Other

researchers predicted the occurrence of *Vibrio cholerae* in Chesapeake Bay in brackish water using temperature and salinity data, while freshwater incidence appeared to be controlled by other factors [Louis *et al.*, 2003]. While the connection between environmental factors and organism abundance is of immediate concern for understanding the ability of *V. cholerae* to cause epidemic disease, these models have all assumed that the isolated bacteria behave identically to the given environmental conditions. Further investigation demonstrates that this is certainly not the case and heterogeneity in isolate genotypes can be linked to distinct changes in phenotype as well.

A spatially intensive 18-month long study of *Vibrio cholerae* distribution in California coastal waters was conducted during 2004 and 2005. More than 75 environmental isolates were confirmed as *Vibrio cholerae* using biochemical screening and PCR amplification of a portion of the 16S-23S intergenic transcribed spacer region [Chun *et al.*, 1999]. The presence and abundance of *V. cholerae* was correlated with more than 30 physical, chemical, and biological measurements including temperature, salinity, turbidity, nutrients, and metals. In addition, a detailed genomic comparison of the isolates was performed using comparative genome hybridization on two types of microarrays constructed from the genome of the fully sequenced *V. cholerae* O1 El Tor strain N16961. The results demonstrate that presence or absence of specific genes based on complete genome hybridization can explain *V. cholerae* phenotypic and genotypic diversity among different ecosystems.

Hybridization profiles for the isolates cluster into three clades based on several sets of contiguous genes that show polarized hybridization signals among groups of isolates. The pattern of clustering appears to be driven by temporal changes, primarily water temperature, leading to high genetic similarity between geographically separated strains isolated during the same season. The genes whose presence and absence varies seasonally is enriched in genes related to chemosensing, cell envelope biosynthesis, and transport and binding of certain substrates. Intuitively, divergence in these genesets makes sense biologically; the ability of the bacterium to interact with its environment is determined by the character of its exterior cell surfaces and sensing of relevant molecules, providing tools for fitness success. Surprisingly, there was a strong seasonal signal in the retention of genes related to the transport and binding of certain carbon sources. Based on functional annotations, isolates from warm weather retained genes for the transport and binding of fructose, while the remaining isolates lacked these genes, but retained genes for the transport and binding of chitosan. As another example, all isolates retained the conserved mannose-sensitive hemagglutinin (MSHA) pilin genes except for divergence coding region for the actual structural mshA pilus. The modified sequence is thought to encode a functional pilus more suited to the aquatic environment as opposed to the human intestine. We investigated how the retention of certain genes was related to both phenotypic differences among strains and variation in environmental parameters measured in the field. In several cases, the presence or absence of collections of genes was directly correlated to dominant or deficient phenotypes. For example, most of the isolates collected during warm weather were missing many of the genes in the metabolic pathway for N-acetylneuraminate degradation. In microcosm experiments, these isolates were also observed to be highly deficient in growth on several derivatives of chitin. Similarly, divisions of isolates exhibiting divergence

in genes related to cell envelope and extracellular polysaccharide biosynthesis displayed different sensitivities to several cell wall inhibitors.

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The ecology and genomics of two marine vibriophages

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Vibrio has been described as the *E. coli* of the seas, most likely because of the ease of isolation of *Vibrio* strains on rich media, and a number of phages have been isolated on these strains. Certainly the most widely studied vibriophages are those that infect strains of *V. cholerae* because of their involvement in the etiology and epidmiology of cholera. The focus of this talk will be vibriophages that infect strains other than *V. cholerae*, with special attention paid to temperate and pseudotemperate phages. Genomic analysis of these phages is hampered by the large percentage of unknown ORFs (only about 25% have any reasonable hit in GenBank, a common problem with marine phages). Although many of these phages participate in lysogenic-like interactions with their hosts, lysogeny genes and modules are missing or undetected. Another common feature is toxicity or virulence-associated genes, which may play a role in host pathogenicity (many of the hosts are fish pathogens). A general overview of the genomes of several vibriophages will be presented. The ecology and genomics of the widely dispersed *V. parahaemolyticus* VP16-like vibriophages will be discussed as well as genomic analysis of the pseudotemperate *Listonella pelagia* phage ϕHSIC.

Regional-scale synchrony of cholera epidemics in the Equatorial Atlantic Coast (Gulf of Guinea)

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Cholera is an ancient disease which had disappeared from most of the developed countries in the last 50 years, but it still persists in many parts of the world with serious epidemics most often localized in tropical areas. This highly contagious disease is due to the bacteria Vibrio cholerae, after ingestion of contaminated water or seafood. V. cholerae is naturally present in the environment and is autochthonous to coastal and estuarine ecosystems. Bacteria are closely associated with many phyto- or zooplankton organisms. Thus any environmental, ecological impact on V. cholerae reservoirs density may have some incidences in bacterial population density. In this context, climatic and/or environmental, ecological changes influencing V. cholerae reservoirs density may be particularly involved in the emergence of cholera in human populations by increasing the probability of contacts between bacterial populations in the ecosystem and riverine human populations. OBJECTIVES: The present work aims at determining and quantifying the relationships between climatic/environmental factors and cholera population dynamics, i.e. the evolution of case numbers with time, across several countries in the Equatorial Atlantic Coast (Gulf of Guinea). METHODS: In a first part, cholera population dynamics is studied using wavelets analysis to characterize the observed dynamics and to synchronism between the different countries. Second, climatic (e.g. S.O.I) and environmental (e.g. Sea Surface Temperature) variables obtained from remote sensing, are also analyzed with similar questions. Finally, results are crossed between environmental variables and disease time-series in order to detect the existence of correlations and phase delays. RESULTS: We observed an important synchrony between cholera epidemics for Benin, Ghana, Republic Demographic of Togo and Nigeria for the period 1987 to 1994, and this was highly synchronized with some climatic and/or oceanographic parameters. CONCLUSION: This comparative approach should allow a better understanding and quantification of the importance of climatic and environmental fluctuations on cholera epidemics in human populations at both the global and regional scales. This work is then a first step toward the objective of building a predictive model for the risk of environmental emergence of Vibrio bacteria in human populations.

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Recent findings on the *Vibrio vulnificus* genome, and on *in situ* gene expression

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Vibrio vulnificus is an estuarine bacterium which causes both food-borne disease and wound infection. Although *V. vulnificus* is commonly found in molluscan shellfish in high numbers, the incidence of disease is relatively low, leading to the hypothesis that not all strains of *V. vulnificus* are equally virulent. Unfortunately, there is currently no easy test to identify virulent strains of this species. We performed DNA sequence data on six clinical and four environmental isolates and found that the strains could be divided into two groups which correlate with clinical (C-type) or environmental (E-type) origin. We designed PCR primers that distinguish between the two groups, and typed 55 randomly selected strains. We found that 90% of the C-type strains were clinical isolates, while 93% of environmental isolates were classified as E-type (1). Thus, the C-type genome is a strong indicator of potential virulence.

Our findings are similar to previous studies on 16S rDNA sequence differences in *V. vulnificus* (2), and the observation that culturability of the two rDNA types varies with water temperature (3). We believe such differences are a consequence of entry into the viable but nonculturable (VBNC) state by these genotypes (for a recent review on the VBNC state in bacteria, see ref. 4). We recently reported that entrance into this state is a consequence of loss of catalase activity in cells exposed to low temperature (5). This loss results in a hypersensitivity to the H2O2 naturally present in complex plating media, and the loss of culturability. We have now confirmed this finding by RT-PCR studies; while *katG* (catalase) expression continues at a high rate in cells incubated at 20°C, it is greatly down-regulated in cells as they enter and persist in the VBNC state. On resuscitation (exposure to 20°C), *katG* expression is up-regulated, and the cells regain culturability. This verifies that loss of catalase activity in VBNC cells is a direct result of decreased transcription of *katG*, and not of decreased activity of the enzyme after its synthesis.

The possibility that the expression observed in VBNC cells is due to longer-lived mRNA rather than *de novo* expression was also addressed. The approximate half-life of *V. vulnificus rpoS* mRNA was determined by inhibiting *de novo* RNA synthesis with rifampin and performing RT-PCR at time points subsequent to treatment. These studies were conducted at both room temperature and 5°C. We found that *rpoS* mRNA in *V. vulnificus* is relatively unstable, as is the case for most other bacterial mRNA species, with a half-life of ca. 3 minutes at room temperature. Transcripts from cells at 5°C, that had been in the VBNC state (<0.1 CFU/ml) for 24h, had a half-life of less than 30 minutes. Because our assay requires 10^3 cells for message detection, our findings indicate that mRNA is being made by metabolically active cells in the VBNC state, and that the mRNA we are detecting was synthesized no more than 30 minutes before RNA extraction. Thus, the gene expression we detect in VBNC cells is due to *de novo* synthesis, and not to an increased stability of mRNA.

In what we believe to be the first studies of the kind, we have also conducted *in situ* gene expression studies by placing log phase cells of *V. vulnificus* into membrane diffusion chambers which are suspended into natural estuarine environments. Genes for an alternate sigma factor (*rpoS*) and an elongation factor (*tufA*) were constitutively expressed by both "E" and "C" type strains in cold estuarine water (*in situ*) as these cells enter the VBNC state (6). However, while expression of the *V. vulnificus* hemolysin gene, *vvhA*, is constitutive in a clinical (C-type) strain, an environmental (E-type) strain exhibited only transient expression of this putative virulence factor, and only following initial exposure to these cold waters. *In situ* studies in warm estuarine waters revealed that, as *V. vulnificus* enters a starvation-survival state, the cells continue to express *vvhA, rpoS* and *tufA* for at least 108h, and *katG* for over 24h, suggesting a need for the continued expression of these genes during this state (7).

V. vulnificus exhibits both encapsulated and non-encapsulated colony morphotypes, the cells of which are virulent and avirulent, respectively (8). Two genes, *wza* and *wzb*, are known to be necessary for capsule production. We have observed that encapsulated cells continue to express these genes *in situ* for at least 24h, providing the first evidence that cells which produce capsules *in vitro* also express capsule genes in the environment. During these studies we noted that, along with encapsulated (*wzb*+) and non-encapsulated (*wzb*-) morphotypes, a third "intermediate" capsular morphotype (*wzb*+) type exists. RT-PCR studies indicate that such cells exhibit greatly reduced expression of *wzb,* with significantly less capsule production *in vitro* compared to the opaque morphotype. Whether such cells are virulent is not yet known.

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- Plenary session 3 -

Genomics

Comparative genomics of *Vibrio cholerae*

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The complete genomic sequence of *Vibrio cholerae* El Tor N16961 was competed in 2000. This genome was found to be 4,033,460 base pairs (bp) consisted of two circular chromosomes of 2,961,146 bp and 1,072,314 bp that together encode 3,885 open reading frames. The vast majority of recognizable genes for essential cell functions (such as DNA replication, transcription, translation and cell-wall biosynthesis) and pathogenicity (for example, toxins, surface antigens and adhesins) are located on the large chromosome. In contrast, the small chromosome contains a larger fraction, and also contains many more genes that appear to have origins other than the gamma-Proteobacteria. The small chromosome also carries a gene capture system (the integron island) and host 'addiction' genes that are typically found on plasmids; thus, the small chromosome may have originally been a megaplasmid that was captured by an ancestral *Vibrio* species. Today, the NIH/ NIAID is sequencing an additional 11 strains of *V. cholerae*,. These data provide an enormous resource for both comparative genomics and a better understanding of this pathogen. These *V. cholerae* genomic sequences provide a starting point for many future studies on this bacterium.

Coming out of the dark: using genomics to shed light on the squid-*Vibrio* symbiosis

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The luminous bacterium *Vibrio fischeri* is the specific symbiont in the light-emitting organ of the Hawaiian bobtail squid, *Euprymna scolopes*. The bacterium provides the squid with luminescence that the animal uses in anti-predatory defenses, while the squid supports the symbiont's nutritional requirements. This association is a useful model to study the molecular basis of specificity and communication in a persistent extracellular colonization of host epithelia. Sequencing of the *V. fischeri* ES114 genome and of *E. scolopes* EST libraries have enabled microarray tool constructions, allowing global gene-expression studies, of both bacteria and host, in this symbiosis. RNA-profile comparisons between bacterial cells isolated from adult squid light organ dissections and bacteria grown in culture revealed differential *in vivo* expression of genes involved in bioluminescence, iron acquisition, motility, and attachment. In addition to these known symbiotic factors, we identified genes encoding functions not previously recognized as involved during symbiosis, including a putative chitin-utilization regulon. Our data suggest that the squid host provides chitin oligosaccharides as energy, carbon, and nitrogen sources for their symbiotic *V. fischeri*. In addition, we have found that chitin oligosaccharides serve as a chemotactic signal that directs bacterial migration to the light organ. These studies exemplify the potential of microarray experiments to increase our understanding of the detailed mechanisms supporting chronic, beneficial infections like those in the squid-*Vibrio* symbiosis.

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Pathogenic mechanism and life cycle of a marine bacterium *Vibrio parahaemolyticus*: an insight from genome sequence

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Vibrio parahaemolyticus, a Gram-negative marine bacterium, is a worldwide cause of food-borne gastroenteritis. Our recent genome sequencing of the clinical *V. parahaemolyticus* strain RIMD2210633 (Lancet 361: 743-749, 2003) identified two sets of genes for the type III secretion system (TTSS), TTSS1 and TTSS2. Here, we constructed a series of mutant

strains from RIMD2210633 to determine whether the two putative TTSS apparatus are functional. The cytotoxicity of mutant strains having a deletion in one of the TTSS1 genes was significantly decreased as compared with that of the parent and TTSS2-related mutant strains. In an enterotoxicity assay using the rabbit ileal loop test, intestinal fluid accumulation was diminished by deletion of the TTSS2-related genes, while TTSS1-related mutants caused a similar level of fluid accumulation as the parent strain. VopD, a protein encoded in the proximity of the TTSS1 region, and a homologue of the *Yersinia* YopD, was secreted in a TTSS1-dependent manner. In contrast, VopP, which is encoded by a pathogenicity island on chromosome 2, and is homologous to the *Yersinia* YopP, was secreted via the TTSS2 pathway. These results provide evidence that *V. parahaemolyticus* TTSSs function as secretion systems, and may have a role in pathogenicity of the organism. The presence of TTSS1 apparatus gene homologues was demonstrated in non-pathogenic *V. parahaemolyticus* and other vibrios such as *Vibrio alginolyticus, Vibrio harveyi,* and *Vibrio tubiashii.* Since the TTSS is a bacterial tool to intimately interact with eukaryotic cells, the results suggest that those vibrios may have a stage/phase in which they intimately interact with certain (but unknown) eukaryotic cells in their life cycle in natural environment.

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Evolutionary dynamics of *Vibrio* integrons

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Integrons are genetic elements capable of the acquisition, rearrangement and expression of genes that are part of gene cassettes. Gene cassettes are the simplest known mobile elements consisting of promoterless genes associated with a recombination site known as a 59 base element (59-be). The integron captures gene cassettes by site-specific recombination, catalyzed by its integrase (intI). A promoter located in the integron allows for the expression of the cassette-associated genes. This system was first discovered through its prominent role in the emergence and dissemination of multiple antibiotic resistances in clinical environments. The sequencing of bacterial genomes fortuitously revealed that these genetic elements could encode many more functions and represent a significant proportion of some bacterial genomes. The most striking examples were Vibrio species, in which gene cassette arrays are very widespread (if not ubiquitous) and can range from 30 Kb (Vibrio fischeri) to over 150 Kb (Vibrio vulnificus) in size. Comparison of cassette arrays we have isolated and sequenced from environmental vibrios to arrays from completely sequenced genomes reveals extensive variability in cassette composition and organization, even between closely related strains of V. vulnificus or V. fischeri. Phylogenetic analysis of the 59-be flanking gene cassettes suggests frequent transfer of cassettes within and between Vibrio species but also outlines the slower rates at which these transfers proceed between more phylogenetically distant relatives. Evolutionary analysis of cassette-

encoded ORFs presents multiple cases of non-specific cassette insertion in chromosomes outside integrons as well as numerous lateral acquisitions from different phyla. The integron integrase genes associated with these arrays suggest that not only the cassettes are mobile, but that the integrons themselves can move between chromosomes within a single species, between different species and can even be co-opted by large plasmids.

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Complete genome sequence of *Vibrio splendidus*: a pathogenic bacterium for a wide range of marine animals.

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Vibrio splendidus is a dominant culturable *Vibrio* in coastal marine sediments, seawater and bivalves. The organism has long been considered as an environmental organism without any pathogenic significance. However for several years, different strains phenotypically related to *V. splendidus* have been associated with mortalities in a wide range of marine animals. The present controversial status of *V. splendidus*, could be attributed to the existence of mutualistic, opportunistic and pathogenic strains within this group (Le Roux et al., 2004).
We initiated sequencing of the *Vibrio splendidus* genome. The strain that we have chosen, LGP 32, is an oyster (*C. gigas*) pathogen, associated to « summer mortalities » syndrome, which has been responsible for high mortality rates in oyster beds in France since 1991. We have started to study the physiology and pathogenicity of those bacteria (Gay et al., 2004a, b) and considered that accessing the complete genome of one of those strains, would be highly beneficial as it would probably accelerate the identification of the virulence determinants and will allow a broader analysis of the *Vibrio* genome plasticity. Indeed, a considerable amount of data concerning human pathogen *Vibrio* species is already available. Moreover, the genomes of four species have already been sequenced, *V. cholerae*, *V. parahaemolyticus V. vulnificus* and *V. fischeri* as well as a *Vibrio*-related species *Photobacterium profondum*. This sum of data renders further comparative genomic studies very likely fruitful.

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Bioinformatic Tools to Compare Vibrio Genomes

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Currently there are six genomes from the *Vibrio* group that have been sequenced and publicly available, (five *Vibrio* and *Photobacterium profundum*). I will give a brief overview of the use of a variety of bioinformatic approaches to compare these 6 genome sequences to each other and to more than 250 additional bacterial genomes. The approaches include: 1.) Chromosome alignment; 2.) AT content in the genome and upstream of genes; 3.) Bias in oligomeric sequences towards the leading or lagging strand of the DNA; 4.) Repeats (local and global); 5.) organization of rRNA operons; 6.) tRNAs and codon usage; 7.) Third nucleotide position bias in codon usage; 8.) Amino acid usage; 9.) Promoter analysis; 10.) Annotation quality; 11.) Blast atlases; and 12.) Proteome comparision, including global regulators, sigma factors, and transcription factors, as well as secreted proteins and secretion systems.

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- Plenary session 4 -

Disease & Epidemiology

Current Concepts in Cholera Dynamics

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A distinctive epidemiological feature of cholera is its appearance with seasonal regularity in endemic areas, such as the Ganges Delta region of Bangladesh and India. Although V. cholerae is a human pathogen, these bacteria constitute part of the normal aquatic flora in estuarine and brackish waters, and water is clearly a vehicle for transmission of V. cholerae. The physical, chemical, and biological parameters which control the concentration of potential epidemic strains in the aquatic environment leading to initiation and conclusion of seasonal cholera epidemics, have been investigated over the years and various hypothesis have been proposed. These include the hypothesis that ecological conditions such as plankton in water are needed to sustain an epidemic. Since V. cholerae can associate with plankton and derive nutrients, environmental factors which support a plankton bloom can lead to a bloom of V. cholerae leading to an epidemic. As environmental factors become unfavourable for the Vibrio choleraeâ€™s plankton host, the epidemic can no longer continue, even though enough bacteria may persist in the waters to strike again during the next cholera season. The role of the human host in selective amplification of V. cholerae from the majority of environmental non-pathogenic V. cholerae prior to an epidemic has also been proposed to be another important factor in cholera epidemiology. More recently, cholera phages in the environment, and their amplification in cholera patients have been shown to remarkably influence the epidemiology of cholera. Over a nearly 3-year period between January 2001 and November 2003 in Dhaka, Bangladesh, the number of cholera patients increased whenever the number of lytic vibriophages in water decreased. Similarly, cholera epidemics tended to end concurrent with large increases in the concentration of these viruses in the water. To understand more about this phenomenon, the dynamics of the V. cholerae-phage interaction was studied during a recent epidemic in Dhaka City. The changing prevalence in the environment of the epidemic V. cholerae O1 strain and a particular lytic cholera phage (JSF4) to which it was sensitive, was measured every 48-72 hours for a period of 17 weeks. The incidence of phage excretion in stools of 387 cholera

patients during the course of the epidemic was also monitored. The peak of the epidemic was preceded by high V. cholerae prevalence in the environment and was followed by high JSF4 phage levels as the epidemic ended. The build up to the phage peak in the environment coincided with increasing excretion of the same phage in the stools of cholera patients. Analysis of representative V. cholerae isolates from cholera stools using DNA probes derived from the phage genome did not show the presence of lysogenic phage genome in the V. cholerae strain. This finding ruled out the possibility that the phage in stools might have originated from induction of lysogenic cells in vivo, and suggested that patients toward the end of the epidemic ingested both JSF4 phage and the outbreak V. cholerae strain. Host-mediated, phage amplification during the cholera epidemic likely contributed to increased environmental phage abundance, decreased load of environmental V. cholerae, and hence, the collapse of the epidemic. Thus, in vivo phage amplification in patients and subsequent phage predation in the environment may explain the self-limiting nature of seasonal cholera epidemics in Bangladesh.

At this point, the roles of environmental factors and plankton, the human host, and the lytic phages all appear to be important, and each represent a part of the intricate mechanism that supports the epidemiology of cholera. Understanding the complex interactions of these different factors and parameters may lead to the development of a unified model to explain cholera dynamics.

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Disruption of quorum sensing protects gnotobiotic *Artemia* from pathogenic *Vibrio harveyi* and *Vibrio campbellii*

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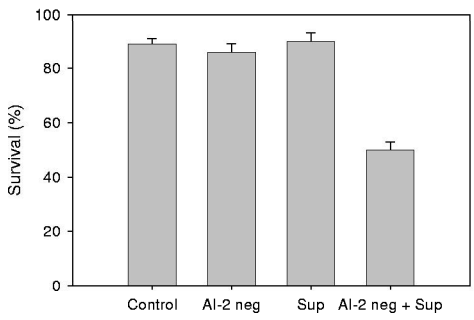
Vibrio harveyi is an important pathogen in the intensive rearing of molluscs, finfish and especially shrimp (Alvarez et al., 1998). Several pathogenic strains phenotypically identified as *Vibrio harveyi* are in fact *Vibrio campbellii*, indicating that this species is also an important cause of disease in aquaculture (Gomez-Gil et al., 2004). The frequent use of antibiotics and disinfectants has lead to the development of resistant strains (Moriarty, 1998). Therefore, there is an urgent need for alternative control techniques. Quorum sensing, bacterial cell-to-cell communication by means of small signal molecules, was shown before to regulate the expression of several virulence-associated phenotypes. Consequently, disruption of quorum sensing has been suggested as a new anti-infective strategy for aquaculture (Defoirdt et al., 2004).

Unlike most Gram-negative bacteria, *Vibrio harveyi* was found to use a multi-channel quorum sensing system. The first channel of this system is mediated by the Harveyi Autoinducer 1 (HAI-1), an acylated homoserine lactone. The second channel is mediated by the so-called autoinducer 2 (AI-2), which is a furanosyl borate diester. The chemical structure of the third autoinducer, called Cholerae Autoinducer 1 (CAI-1) is still unknown. All three autoinducers are detected at the cell surface and activate or inactivate target gene expression by a phosphorylation/dephosphorylation cascade (Henke and Bassler, 2004b). Phenotypes that were found to be controlled by this quorum sensing system include bioluminescence (Bassler et al., 1993) and the expression of several virulence factors such as a type III secretion system (Henke and Bassler, 2004a), extracellular toxin (Manefield et al., 2000) and a siderophore (Lilley and Bassler, 2000). However, as most pathogens usually use several different virulence factors, it is not always clear what the impact is of one single virulence factor on the virulence of the pathogen.

We investigated the impact of quorum sensing on the virulence of *Vibrio harveyi* by performing *in vivo* challenge tests with gnotobiotically grown *Artemia franciscana*. Sterile *Artemia* nauplii, obtained by hatching of decapsulated cysts, were cultured in filtered and autoclaved artificial seawater and fed dead LVS3 bacteria. Working in the absence of other bacteria is important for these types of experiments to avoid artefacts. Indeed, AI-2-mediated signalling was found to be present in many different species.

Previously, a series of challenge tests with wildtype *Vibrio harveyi* BB120 and quorum sensing mutants that were derived from this strain revealed that mutations in the AI-2-mediated channel of the *Vibrio harveyi* quorum sensing system abolished virulence of strain BB120, whereas mutations in the HAI-1-mediated channel had no effect on its virulence (Defoirdt et al., 2005). Furthermore, we investigated whether an exogenous source of AI-2 could restore the virulence of an AI-2 non-producing mutant. Filter-sterilised supernatant of an AI-2 producing *Vibrio harveyi* culture was chosen as an exogenous source of AI-2 because when we did our experiments, the molecule was not available in a purified form. The supernatant could indeed restore the virulence of the AI-2 non-producing mutant (Fig. 1).



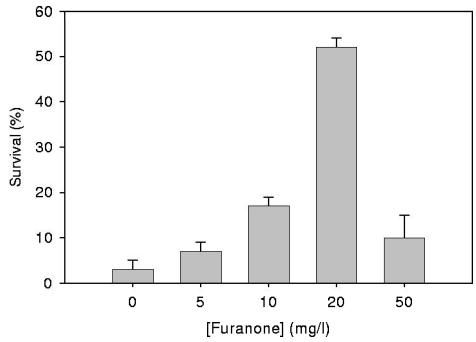


Fig. 1: Percentage survival of *Artemia* after 48 h challenge with the *Vibrio harveyi* AI-2-negative mutant; with and without the addition of supernatant (as an exogenous source of AI-2). The error bars represent the standard error of four repeats. **Fig. 2:** Percentage survival of Artemia after 48 h challenge with *Vibrio campbellii* LMG21363, with and without the brominated furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. The error bars represent the standard error of three repeats.

Subsequently, different pathogenic *Vibrio harveyi* and *Vibrio campbellii* isolates were screened for the production of autoinducers by using *Vibrio harveyi* reporter strains. Cell-free culture supernatants of the isolates significantly induced bioluminescence in the reporter strains, indicating that all isolates produced all three types of autoinducers (HAI-1, AI-2 as well as CAI-1).

Finally, the effect of a quorum sensing disrupting brominated furanone on the virulence of the pathogenic *Vibrio harveyi* and *Vibrio campbellii* isolates was investigated in our model system with gnotobiotic *Artemia* nauplii. The result for the virulent isolate *Vibrio campbellii* LMG21363 is represented in Fig. 2. The addition of the furanone, at 20 mg/l, significantly enhanced the survival of *Artemia* nauplii challenged to the isolates, although no complete protection was obtained for some of the *Vibrio campbellii* isolates. Unfortunately, the furanone also showed to be toxic to the brine shrimp since high mortality occured in the treatment with 50 mg/l of furanone. We plan further experiments with synthetic derivatives of the furanone and with compounds that target the AI-2 synthase enzyme LuxS.

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Iron Transport and Virulence of the Fish Pathogen *Vibrio anguillarum*

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One proven virulence factor for serotype O1 strains of the fish pathogen *Vibrio anguillarum* is a very efficient iron-sequestering system encoded by the 65-kilobase pairs (kb) pJM1 plasmid. Our laboratory has demonstrated that this iron sequestering system consists of the siderophore anguibactin and a specific transport complex for the ferric-siderophore which are essential for the pathogenicity of this bacterium. From the structure of the siderophore and genetic, biochemical and in silico studies of biosynthetic genes identified on the pJM1 plasmid from *V. anguillarum* strain 775, a pathway for anguibactin biosynthesis mediated by nonribosomal peptide synthetases has been proposed. The anguibactin precursors DHBA and L-cysteine are activated by the adenylation (A) domains of the AngE protein and the AngR protein respectively. The next step requires the phosphopantoheynylation by AngD of both the ArCP of the AngB/G protein and the PCP domain of the AngM protein. The AngE-activated DHBA is tethered on the phosphopantheteinlate arm (pPant) of the ArCP of the AngB/G protein while activated cysteine is tethered to the pPant of the PCP domain of the AngM protein. The AngR protein also posses a PCP domain on which cysteine could be tethered but the PCP domain of AngR is not functional because an essential serine is replaced by an alanine. The C domain of AngM catalyzes the formation of the peptide bond between DHBA and cysteine. In this or later steps of anguibactin biosynthesis, cysteine is cyclized to a thiazoline ring by AngN, which possesses two Cy groups. Next, the dihydroxyphenylthiazoline dipeptide is released from the PCP domain of AngM resulting in the free anguibactin molecule. N-hydroxyhistamine is obtained by modification of histidine catalyzed by two tailoring enzymes, the mono-oxygenase AngU and the histidine decarboxylase AngH. Ferric-anguibactin is transported into the cell cytosol by the FatABCD complex. The genes encoding these proteins

are located on the virulence plasmid and transcribed as one polycistronic message with the biosynthesis genes *angR* and *angT* (ITB operon). The ferric-anguibactin receptor FatA is an 86-kDa protein that is essential for anguibactin transport. The FatA amino acid sequence has a TonB box at its amino terminal end. This TonB box is one of the interaction sites between the outer membrane receptor and the energy transducer TonB. *V. anguillarum* has two *tonB* systems; *tonB1* in chromosome 2 and *tonB2* in chromosome 1. Transport of ferric anguibactin only requires the TonB2 protein. Once in the periplasm the ferric-anguibactin is bound by the periplasmic binding protein FatB, a 35-kDa lipoprotein that is anchored in the inner membrane. FatB shuttles ferric-anguibactin to the permeases FatC and FatD in the inner membrane that internalize the ferric-anguibactin complex to the cytoplasm, using the energy generated by ATP hydrolysis. In *V. anguillarum* the plasmid-mediated iron uptake system is negatively regulated by the chromosomally-encoded Fur. Two positive regulators, the AngR protein and products encoded in the Transacting factor (TAF) region, were shown to act at the same promoter. Thus, AngR is a bi-functional protein that besides its role in biosynthesis is also involved in regulation. The TAF product is encoded in a region of the virulence plasmid non-contiguous to the ITB operon.. Evidence exists that the relative expression of the ang genes within this operon is regulated by an antisense RNA, RNAβ. The opportunistic human pathogen *Acinetobacter baumannii* 19606, which causes severe infections in compromised patients, produces the siderophoroe acinetobactin. Interestingly, this catechol siderophore is highly related to anguibactin. The only difference between these two iron-scavenging compounds is that anguibactin contains cysteine while threonine is the amino acid found in acinetobactin as a functional group. Siderophore utilization bioassays showed that acinetobactin enhances the growth of *V. anguillarum* under iron-stress conditions. A predicted outer-membrane protein receptor for ferric acinetbactin, named BauA, was highly similar to FatA, the receptor for ferric anguibactin. and they are immunologically and functionaly related. The *bauA* gene is part of a polycistronic locus that includes the *bauDCEB* coding regions, which has the same arrangement of the *fatDCBA* locus with the exception of the presence of *bauE*, which is related to the ATPase component of Gram-positive ATP-binding cassette (ABC) transport systems. This entire locus is flanked by genes encoding predicted proteins related to AngU and AngN, *V. anguillarum* proteins required for the biosynthesis of anguibactin. Taken together, these results demonstrate that these two pathogens, which cause serious infections in unrelated hosts, express very similar siderophore-mediated iron-acquisition systems. Transformation, conjugation and transposition occur in Acinetobacter and the pJM1 iron-uptake genes are flanked by insertion sequences in a composite transposon-like structure. Furthermore, Acinetobacter is a component of the bacterial flora of salmonid fishes. Thus, plasmid conjugation, transformation and/or transposition might have played a role in the transmission of these essential genes between these two unrelated bacterial strains present in the microbial flora of salmon and trout.

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The *Vibrio cholerae* flagellar regulatory hierarchy controls expression of virulence factors

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Vibrio cholerae is a gram-negative bacterium responsible for the disease cholera. It is highly motile and possesses a single polar flagellum. The expression of the flagellar genes is tightly regulated by a flagellar transcription hierarchy. We constructed *V. cholerae* strains containing mutations in the major flagellar regulatory genes (*rpoN, flrA, flrC*, and *fliA)* that control the flagellar transcription hierarchy, and performed microarray experiments to analyze their transcription profiles. Results revealed that the *V. cholerae* flagellar transcription hierarchy is organized essentially exactly as previously postulated (Prouty et al, *Mol. Microbiol.***39**:1595), and also revealed a number of additional genes both positively and negatively regulated by the flagellar regulatory cascade. Genes with proven or putative roles in virulence (e.g. *ctx*, *tcp*, hemolysins) were generally upregulated in the flagellar mutants, consistent with the previous hypothesis of an inverse relationship between motility and virulence (Gardel and Mekalanos, *Infect. Immun.***64**:2246). Promoter-*lacZ* analysis confirmed the microarray results of reduced expression of two genes encoding GGDEF (diguanylate cyclase) proteins and a gene predicted to encode the outer membrane component (TolC homologue) of a Type I secretion system.
Flagellar regulatory mutants show decreased hemagglutination and increased hemolysis of human type O red blood cells. Mutation of one of the flagellar-dependent GGDEF genes also caused decreased hemagglutination, and complementation with the plasmid-encoded GGDEF gene restored hemagglutinating activity to GGDEF and flagellar mutant strains, suggesting that the flagellum might regulate the expression of this hemagglutinin via the GGDEF protein.
Strains with a deletion in the Type I secretion OM protein also show decreased hemagglutination, suggesting that the hemagglutinin is a substrate of this secretion system. Mutation of one of the up-regulated hemolysin genes (*tlh*) in flagellar regulatory mutants abolished hemolysis, thus identifying the flagellar-regulated hemolysin.
Our results indicate that the flagellar regulatory genes regulate the expression of a number of non-flagellar genes, including virulence factors. This regulation is likely to be complex, and includes mechanisms involving cyclic diguanylate and Type I secretion.

Vibrio cholerae and Lipopolysaccharide: Analysis of the O Antigen Transfer

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As for other pathogenic bacteria, O polysaccharides serve important biological functions in disease for *V. cholerae*. They protect against the host immune recognition system, complement attack, the immune response, and they are probably involved in mediating adherence to host surfaces. The majority of Gram-negative bacteria transfer O antigen polysaccharides onto the lipid A-core oligosaccharide via the action of surface polymer:lipid A-core ligases (WaaL). To learn more about this complex function, we characterised several gene functions, among them were the WaaL proteins of *Vibrio cholerae* with emphasis on structural and functional characterisation of O antigen transfer and core oligosaccharide recognition. We demonstrate that the activity of two distantly related O antigen ligases is dependent on the presence of N-acetyl-glucosamine, and substitution of an additional sugar, i.e. galactose, alters the site specificity of the core oligosaccharide necessitating discriminative WaaL types. Protein topology analysis and a conserved domain search identified two distinct conserved motifs in the periplasmic domains of WaaL proteins. Site directed mutagenesis of the two motifs, shown for WaaLs of *Vibrio cholerae* and *Salmonella enterica*, caused a loss of O antigen transfer activity. Moreover, analogy of topology and motifs between WaaLs and O polysaccharide polymerases (Wzy) reveals a relationship between the two protein families, suggesting that the catalysed reactions are related to each other.

An overview of the pathogenesis of *Vibrio aestuarianus* strain 01/32 to the Pacific oyster *Crassostrea gigas*

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In France, shellfish production is a traditional well-established industry, mainly based on commercial *Crassostrea gigas* oyster farming. Since 1991, high mortality rates of *C. gigas* spat (60 to 100%) have been reported during summer, both in the field and in hatcheries, and

are of major concern to oyster farmers. Several studies on this subject hypothesized that recurrent oyster mortality could be the result of complex interactions between the physiological and/or genetic status of the host, numerous environmental factors, and one or more infectious agents. In this context, a bacterial strain was isolated from hemolymph of moribund *C. gigas* juveniles, sampled during a summer mortality outbreak occurring in an experimental hatchery. The strain was identified by phenotypic and genotypic methods as *Vibrio aestuarianus* and was given the designation 01/32. The pathogenicity of this isolate was previously assessed in our laboratory by experimental challenges, resulting in high *C. gigas* mortality rates (60-90 %). However, mechanisms involved in the pathogenicity of this strain are not yet well defined. Furthermore, bacteria-induced host tissue alterations, as well as immune responses, remain poorly understood in oysters compared to the studies on the pathogenic processes for vertebrates and plant pathogens. The purpose of this presentation is therefore to propose a synthetic overview of the studies on the pathogenesis of *V. aestuarianus* strain 01/32 to the oyster *C. gigas*.

To establish an infection model for *V. aestuarianus* 01/32, we first studied during the time course of an experimental infection the oyster cellular immune responses using flow cytometric-based hemocyte assays, and the protease activity of hemolymph. Following infection by *V. aestuarianus* 01/32, a significant inhibition of host hemocyte phagocytosis and adhesive capabilities was noticed over the experimental period, while a strong stimulation of their oxidative metabolism occurred. This suggested that the pathogen might evade the oyster immune system by impairing hemocyte functions. Additionally, a significant increase in protease activity was observed in hemolymph samples of infected-oyster. Gelatin-impregnated gel electrophoresis revealed the presence of additional proteases, corresponding to *V. aestuarianus* extracellular products (ECPs). The contribution of these ECPs to the pathogenesis was consequently determined. ECPs displayed lethality to animals, with a LD50 value of 3.3 µg/g body weight. Moreover, *in vitro* treatment of hemolymph samples with ECPs caused the same immunosuppressant effects on oyster hemocytes as those induced during infection (i.e. cell morphological modifications as well as alterations of hemocyte functions). These results indicated that *V. aestuarianus* 01/32 secretes one or more factors which might play an important role in the pathogenicity. We therefore focused on the characterization of these extracellular products. The ECPs displayed a strong protease activity, which was dramatically reduced by chelating agents EDTA and 1,10-phenanthroline, suggesting that the enzymatic activity was probably linked to a metalloprotease. Moreover, *in vivo* studies revealed that the lethal effect to animals could be strongly decreased by exposition of ECPs to chelating agent 1,10-phenanthroline. Additionally, flow cytometric-based assays demonstrated that the hemocyte alterations induced by ECPs were also reversed to near-control levels by titration with 1,10-phenanthroline. This metalloprotease-like enzyme seems therefore strongly implicated in the disease process. A metalloprotease gene (vam) was recently cloned from *V. aestuarianus* 01/32, showing an open reading frame of 1836 nucleotides and encoding a putative 611 aa polypeptide. Comparative analyses of the deduced aa sequence of *vam* gene exhibited high homologies with other *Vibrio* metalloproteases, especially with EmpA from *V. anguillarum* (84 % identity). High performance liquid chromatography procedures are currently performed on ECPs to purify the corresponding metalloprotease and to elucidate its role in the virulence.

To conclude, our results, and data from the literature, led us to design a model for *V. aestuarianus* 01/32 pathogenesis, which will be briefly discussed as future prospects.

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Identification and characterization of two hemolysin gene clusters in *Vibrio anguillarum*.

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Vibrio anguillarum is a causative agent of vibriosis in fish. The distribution of vibriosis is worldwide, causing great economic loss to the aquaculture industry. Hemolytic activity has been suggested as a virulence factor during infection of fish by *V. anguillarum* by contributing to hemorrhagic septicemia and diarrhea. In order to identify and characterize the hemolysin genes, a mini-Tn*10*Kan mutagenesis clone bank of *V. anguillarum* was screened. While no hemolysin negative strains were observed, several mutants with 2-3-fold increased hemolytic activity were found. The region containing the insertion mutation was cloned, sequenced, and found to contain the *V. anguillarum* hemolysin gene (*vah1*) and two other open reading frames, a putative lactonizing lipase (*llpA*) and a putative phospholipase (*plp*). The mini-Tn*10*Kan was inserted into *plp*. Site-directed mutagenesis of each gene revealed that mutations in *vah1* and *llpA* did not affect hemolytic activity, but insertions into *plp* caused a 2-3-fold increase in hemolysis. Double mutations in *plp* and either *vah1* or *llpA* resulted in wild-type hemolytic activity. Complementation of *plp* restored hemolytic activity to wild type levels. Spectrophotometric determination of hemolysin specific activity demonstrated that activity on a per cell basis peaked during the first 2 hours of growth in Luria Broth + 2% NaCl (LB20). Real-time quantitative reverse transcriptase PCR revealed that in *V. anguillarum* wild-type strains M93Sm and NB10 transcription of *plp* and *vah1* peaked at 2 h of growth in LB20. Additionally, expression of *vah1* measured in the *plp* mutant strain, JL01 during the first 2 h of growth was >8 times higher than in M93Sm. Additional rounds of mini-Tn*10*Kan mutagenesis performed on the *vah1* mutant strain JR1 (*vah1*::pNQ705-vah1) resulted in a hemolysin negative mutant. The region containing the insertion mutation was cloned, sequenced, and found to contain a putative *rtxACBD* gene cluster. RTX (repeats in toxin) hemolysins are found in many Gram-negative bacteria and usually include genes encoding the toxin (*rtxA*), an acylase (*rtxC*), and toxin transporters (*rtxB* and *rtxD*). The mini-Tn*10*Kan was inserted into *rtxB*. nfection studies were carried out in juvenile Atlantic salmon (*Salmo salar*) to compare the effects of mutations in *plp*, *vah1*, *llpA*, and *rtxB* upon virulence. Mutations in *plp* and *llpA* did not affect virulence of *V. anguillarum*. The mutation in *vah1* decreased virulence to about 25% of that observed in the wild type strain. Juvenile Atlantic salmon infected by a *V. anguillarum* strain containing mutations in both *vah1* and *rtxB* suffered about 10% of the mortalities exhibited by salmon infected with the wild type parental strain. The data strongly suggest that several genes located in two gene clusters encode hemolytic activity. One gene

cluster includes *plp*, *vah1*, *llpA* and *llpB*. The second gene cluster includes genes homologous to *rtx* genes found in other *Vibrio* species - *rtxACBD*. The data also suggest that *plp* acts as a negative-regulator of *vah1* and *llpA*.

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- Poster abstracts -

Biodiversity & Taxonomy

B-1

Signaling by a GGDEF/ EAL – containing protein mediates regulation of gene expression in *V. parahaemolyticus*

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V. parahaemolyticus is a marine bacterium and pathogen that adapts to communal life on surfaces through changes in cell morphology. On surfaces it can differentiate to a swarmer cell by turning on the expression of lateral flagellar genes. The *scrABC* operon acts as a positive regulator of swarmer cell differentiation and a negative regulator of capsule production. Mutation of *scrABC* reduces swarming and induces a crinkly colony morphology. Overexpression of *scrABC* induces expression of lateral flagellar genes in liquid culture and decreases transcription of *cpsA*, the capsule-encoding locus. The operon consists of three genes: *scrA* encodes a potential pyridoxal-phosphate-dependent enzyme, *scrB* encodes a potential extracellular solute-binding protein and *scrC* encodes a potential sensory protein. The ScrC N-terminus has two predicted transmembrane domains flanking a potential periplasmic region, and the C-terminus contains putative GGDEF and EAL domains. These two conserved domains, named after signature amino acid motifs, are found in diguanylate cyclases and phosphodiesterases and are thought to synthesize and degrade cyclic diguanosine monophosphate (cyclic-diGMP). A role for cyclic-diGMP is being described in an expanding list of organisms where it has been implicated in regulating production of diverse cell surface molecules. In order to dissect the mechanism of regulation of swarming and CPS production by ScrABC, luminescence (*lux*) and β-galactosidase (*lacZ*) reporter strains were used. The luminescent reporter strain contained an insertion of the *lux* operon within the gene encoding the lateral flagella hook (*lfgE*). Therefore, the strain produced light when grown on surfaces but not in liquid culture. The *lacZ* reporter strain contained a fusion in the *cpsA* locus. Both reporter strains also contained a deletion of the chromosomal *scrABC* locus. To probe function of *scrC*, it was cloned singly and as part of the *scrABC* operon into an IPTG-inducible expression vector. We analyzed effects of these plasmids on lateral flagellar and *cps* gene expression, colony morphology, biofilm formation, and cyclic-diGMP levels. Overexpression

of *scrC* had the opposite effect as of overexpression of *scrABC* in all tests performed. ScrC acted as a repressor of swarming and *laf* transcription and acted as an inducer of the crinkly colony morphology, *cps* transcription and biofilm formation. The activity of ScrC was completely reversed upon the co-production with ScrA and ScrB. ScrABC activated *laf* transcription, while inhibiting biofilm formation and *cps* production. Examination of cellular nucleotide pools revealed that ScrC, in the absence of ScrA and ScrB, acted as a cyclase to produce high levels of cyclic-diGMP. A deletion engineered into the *scrC* gene created an allele that produced a truncated ScrC with only the GGDEF domain (ScrCΔEAL). This deletion had no effect on the transcriptional regulatory activities and diguanylate cyclase ability of ScrC; thus, the GGDEF domain was sufficient to produce cyclic di-GMP, diminish lateral flagellar transcription, and induce *cps* gene expression. However, the EAL domain was critical for function of ScrC in the context of ScrA and ScrB. Loss of function of the EAL domain reversed the transcriptional regulatory effects (e.g., converting ScrABC from an activator to a repressor of lateral flagellar genes) and greatly increased the cellular cyclic-diGMP concentration. These results show that: 1, cyclic-diGMP levels inversely modulate swarming and capsular polysaccharide gene expression; 2, ScrC has both the capacity to form and degrade cyclic-diGMP; 3, the EAL domain is not required for cyclic-diGMP production; 4, ScrA and ScrB convert ScrC from a cyclase to a phosphodiesterase; and 5, the EAL domain is essential for activity of ScrC in the context of ScrA and ScrB. This data is consistent with a model in which activity of ScrC is modulated by ScrA and ScrB. We propose that environmental signaling, acting through the periplasmic binding protein ScrB, influences the activity of ScrC and hence the pool of cyclic di-GMP, which in turn affects gene expression. The *scrABC* operon participates in controlling the decision to be a highly mobile swarmer cell or a more adhesive, biofilm-proficient cell type. The nature of the specific environment cues and how the small signaling molecule affects gene expression remain to be discovered.

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B-2

Genotypic and Phenotypic Characterization of *Vibrio parahaemolyticus* isolated from Molluscs at Harvest and from Clinical Sources in Canada.

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Objective:

A surveillance project was pursued to examine the relationship between *Vibrio parahaemolyticus* strains isolated from environmental (molluscan shellfish) and clinical sources in Canada in order to (i) assess the risk associated with this microorganism and (ii) make scientifically sound decisions to mitigate any risk associated with consumption of raw molluscs.

Background:

Human illness due to *Vibrio* species is a concern globally, but with regional variations. Morbidity and mortality in humans, such as wound infection, gastroenteritis, cholera and primary septicemia, are caused by the food-borne and/or water-borne biotypes. *Vibrio parahaemolyticus* (Vp) is a halophilic vibrio known to cause human infections with three major clinical syndromes: gastroenteritis, wound infections and septicemia. It is a major cause of gastroenteritis worldwide, and is known to comprise at least three pandemic strains. Vp and other halophilic vibrios are ubiquitous in the estuarine environment, particularly in temperate regions, and have been detected in coastal waters of the Americas. In temperate regions, seasonal occurrences have been reported, the majority being in the warmer months of the year. In subtropical regions such as Florida (US), illnesses can occur year-round due to the consumption of raw or undercooked shellfish, such as oysters, clams and other seafood products. Gastroenteritis due to Vp is associated with the ingestion of contaminated shellfish and is characterized by severe cramping, abdominal pain, vomiting and watery to bloody diarrhea. The illness is usually self-limiting and requires restoration of water and electrolytes. Immunocompromised individuals are at a higher risk. Several outbreaks have occurred in North America, including a major one in 1997 that involved more than 400 people in several (US) states as well as British Columbia (Canada).

Methodology:

In this study, phenotypic and genotypic characterization of Vp isolates from environmental molluscs harvested from selected Canadian sites and clinical sources in Canada was undertaken. Molluscan shellfish were harvested from sites in the coastal waters of British Columbia (B.C.) and the Gaspé Peninsula and Îles-de-la-Madeleine in Québec between May and October (2002 to 2004). Phenotypic characterization was based on biochemical profiles using standard diagnostic kits, such as API-20E (Analytical Profile Index derived from twenty biochemical reactions). Molecular characterization was based on ribotyping and presence/absence of species-specific hemolysin(s) by polymerase chain reaction (PCR) based assays. Ribotyping was carried out using the Qualicon (Delaware, USA) Riboprinter microbial characterization system according to the manufacturer's instructions. The resulting riboprint patterns were recorded and analysed using the software supplied with the Riboprinter system.

Results:

During this three-year period 35 / 59 (59%) and 9 / 42 (21%) of the samples from western and eastern coastal waters, respectively, were positive for Vp. Levels of Vp ranged from 2 to 4 log CFU/g in oysters from the west coast and less than 3 log CFU/g in clams and mussels from the Gaspé region. The occurrence of Vp was highest in the midsummer months. Clinical strains were mostly obtained from medical facilities in B.C., and a few from the United States, and all were associated with oyster consumption. From a total of 107 Vp strains, comprising two standard strains, 61 environmental and 44 clinical isolates, 49 ribogroups and 19 different biochemical profiles were identified. Certain ribogroups contained both clinical and environmental isolates, indicating that some ribotypes may be of concern for human health. Similar clustering was also observed with some biochemical profiles.

Inferences:

Diversity within a biotype is well known for Gram-negative bacteria and the fact is evidenced by the presence of several serovars of Vp in the world. The majority of Vp clinical strains produce a heat-stable hemolysin (TDH) that causes lysis of human red blood cells and are identified as Kanagawa phenomenon positive (KP+) strains. Some KP negative strains, however, have been found to cause human illness by virtue of other virulence traits. A thermolabile hemolysin (TLH) is known to be present in all Vp strains, therefore, presence of the gene (*tlh*) has been used for species identification after initial biochemical screening. In the environment, around 1% of TLH positive strains are known to carry the pathogenic *tdh* marker. The widespread occurrence of Vp in molluscs from Canadian coastal waters indicates that aquacultural and feral molluscs present a potential human health risk if consumed without adequate precautions.

Impact:

This preliminary data and subsequent analyses will facilitate the development of policies and guidelines pertaining to the safety of molluscan shellfish.

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B-3

Diversity of *Vibrio* species associated with the cultured Galician marine organisms

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A polyphasic identification of culturable aerobic bacteria associated with reared Galician marine organisms using numerical taxonomy, ribotyping and sequencing of 16S rRNA gene was performed.
This research allowed the identification of closely phylogenetic *Vibrio* species included in *V. splendidus-V. lentus* related group as *Vibrio tasmaniensis*, *V. pomeroyi*, *V. lentus* and *V. splendidus*. *V. neptunius, V. icthyioenteri, V. scophthalmi, V. mediterranei, V. aestuarianus, V. halioticoli, V. parahaemolyticus* and *V. fischeri* were also identified.
The phenotype of those *Vibrio* species was analysed by comparing the phenotypical results of the strains previously identified by molecular methods and with those of closely related type strains. Differential traits of phenotypical identification of that species were selected in order to make an easy identification table for rapid biochemical identification. Primers selected from the variable sequence of 16S rRNA of these species were tested in order to find the adequate PCR conditions for a rapid specific molecular identification. This study endorses the use of both molecular and phenotypical techniques for improving the identification of *Vibrio* species.

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B-4

Biodiversity of *Vibrio cholerae* Strains Isolated in Russia: Genomic Structure and Molecular Typing

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The seventh cholera pandemic, caused in 1961 by *Vibrio cholerae* El Tor biovar, is still going on collecting human victims in many countries of the world. Large cholera epidemic outbreaks were registered in the territory of the Russian Federation in various years (1970-1975, 1994, 1999, 2001). Sequencing of the whole genome of *V. cholerae* capable to live in two ecological niches (human organism and water ecosystems), demonstrated the existence of two

chromosomes within the cells differing in their sizes and functions. Genomic instability of this pathogen is called forth by the presence of various mobile genetic elements (MGE) involving the genes of virulence, pandemicity and persistence (CTX and RS1 prophages, pathogenicity islands VPI-1 and VPI-2, pandemic islands VSP-1 and VSP-2, persistence island EPI) in the large chromosome. To study V.cholerae genetic variability, we tested 175 natural strains isolated from different sources in different years, for the presence of 20 genes associated with virulence, pandemicity and persistence using mono- and multiplex polymerase chain reaction (PCR). Of the 20 genes under test, 4 were localized in the core chromosomal region: toxR, global regulatory gene, rtxA determining toxin RTX biosynthesis, hapA coding for soluble hemagglutinin-protease, attRS, site for the insertion of CTX and RS1 phages into the chromosome. The presence of various MGE in the chromosome was detected using PCR analysis to reveal the following 16 genes: ctxA, zot and ace included into the core region of CTX prophage genome; rstC, localized in RS1 prophage; aldA, mop, tcpA, toxT and hel1760, nanH, rep1803 located in the central and peripheral fragments of VPI-1 and VPI-2, respectively; vc0175, vc0183, and vc0490, vc0496 genes of VSP-1 and VSP-2 peripheral fragments, respectively. PCR testing of 86 clinical isolates demonstrated 78 of them (90.7%) to contain the whole gene set, irrespectively of the place and time of their isolation. On the other hand, 8 strains (9.3%) were deprived either of ctxA gene (5.7%) coding for cholera toxin (CT) and making part of CTX prophage, or VSP-1 and VSP-2 (3.6%). When ecological niches alternate (human organism-environment) during cholera outbreaks, virulent strains are likely to undergo adaptive modifications expressed in the loss of MGEs. Of the 20 strains isolated from water during the cholera epidemics, only 8 ones (40.0%) were containing all the MGE under study. CTX prophage was shown to be deficient in 4 of these (20.0%) because it lacked either ctxA, or both ctxA and zot genes, or it contained none of the three genes, ctxA, zot, ace. Retention of gene tcpA coding for TCP (a main colonization factor and phage CTX receptor) is indicative of the possibility for them to acquire epidemic potential as a result of phage conversion. A non-toxinogenic strain has also been detected whose tcpA gene was homologous to that of V.cholerae classical biovariant, as confirmed by sequencing analysis. The rest of the isolates studied (40.0%) were deprived of all the MGEs, except EPI. During the inter-epidemic period, three groups of strains (56 isolates) were obtained from water: single virulent strains, that retained all MGE (1.8%); those carrying but individual genes or virulence gene blocks (16.1%); and isolates that had conserved only genes of the core chromosomal region (82.1%). RAPD-PCR typing procedure showed the clinical strains to be a homogenous group suggesting their clonal origin. Vibrio strains retaining only separate virulence genes in their genome seem to be derivatives of epidemically hazardous clones. Avirulent "water" vibrios formed a heterogenous group demonstrating no apparent phylogenetic associations with the former two groups. Thus, the observed wide genetic diversity and variability of V. cholerae strains isolated in a cholera non-endemic territory during epidemically safe and unsafe periods, should be considered as a strong stimulus to carry out continuous monitoring of the environments as part of epidemiologic surveillance activities.

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B-5

Characterisation of a natural population of Vibrio sp. using MLSA

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Vibrios are known to form intimate symbiotic and parasitic associations with a vast range of eukaryotic hosts, although little is known about how the precise role of host specialisation in generating and maintaining genomic diversity. Here we address this issue on both local and global scales by sampling >200 *Vibrio* isolates from sea anemones, belonging to the genus *Actinia*, from rocky shore sites in South Wales, UK, and Auckland, NZ. We have characterized these strains, which putatively reflect a single ecological niche, by sequencing in multiple protein encoding loci (MLSA). The data suggest some evidence of ecological adaptation, but also remarkably high rates of horizontal gene transfer. These data are also relevant for further resolving the phylogenetic relationships of this genus.

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B-6

Rapid identification of marine luminescent bacteria by amplified ribosomal DNA restriction analysis

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Luminescent bacteria are grouped as a functional unit on the basis of their ability to emit visible light, and they possess the *lux* genes for light production. These bacteria are ubiquitous in the marine environment and isolated from seawater, sediment, and detritus in shallow coastal and deep pelagic water. They often associate with living and nonliving marine organisms. Certain luminescent species are established as species-specific symbionts with marine fish and squids. All reported species of luminescent bacteria belong to the class *Gammaproteobacteria*. Among 17 luminescent species currently known, 14 are marine bacteria: nine *Vibrio* species and three *Photobacterium* species of the family *Vibrionaceae* and two *Shewanella* species of the family *Alteromonadaceae*.

Identification to the species level is indispensable for ecological studies of marine luminescent bacteria. Although conventional phenotypic characteristics have been used commonly in

taxonomic identification, an alternative molecular approach, an identification method based on luciferase gene (*lux*A) sequences has been developed[2,4]. However, species identification based on *lux*A gene sequences may not always be successful, mainly because *lux*A genes are highly divergent even within the same species. To clarify the ecology and diversity study of luminescent bacteria in the marine environment, there is a need to create a simple and rapid identification method for natural isolates.

We describe here a method to rapidly identify marine luminescent bacteria: amplified ribosomal DNA restriction analysis (ARDRA). ARDRA is based on the restriction patterns of 16S rDNA digested with five enzymes (*Eco*RI, *Dde*I, *Hha*I, *Hinf*I, and *Rsa*I) and clearly distinguished the 14 known species of marine luminescent bacteria. Combinations of the patterns of two enzymes, *Hha*I and *Rsa*I, allowed discrimination of seven species of luminescent bacteria: *Photobacterium angustum*, *Vibrio cholerae, V. fischeri, V. logei, V. mediterranei, V. orientalis,* and *V. splendidus*. Although these two enzymes did not allow us to discriminate *V. harveyi* from *V. vulnificus*, ARDRA with *Hinf*I allowed clear distinction between the two strains. Similarly, another ARDRA with *Eco*RI allowed us to discriminate between *P. leiognathi* and *P. phosphoreum*, and *Dde*I differentiated *S. hanedai* and *S. woodyi.*

To elucidate luminescent bacterial diversity in the Japanese coastal environment, we applied ARDRA to 70 isolates derived from seawater, sediments, and copepod samples collected in Sagami Bay, Japan. Of the isolates, 68 were grouped into ARDRA patterns similar to those of the six known luminescent species, *Photobacterium angustum*, *P. leiognathi, P. phosphoreum, Shewanella woodyi, Vibrio fischeri,* and *V. harveyi*. Among them, *P. leiognathi, P. phosphoreum, V. fischeri,* and *V. harveyi* have been reported to contribute the majority of culturable luminescent bacterial population in the ocean[3]. *Photobacterium angustum* had been known as a nonluminescent species in this genus, but recently this species was found to contain luminescent strains isolated from Sea of Cortes, Mexico[1]. *Shewanella woodyi* is first described in 1997 as luminescent isolates from the Alboran Sea. Since then, no report of the species has been published. Our ARDRA results strongly suggested that *P. angustum* with luminescent phenotype and *S. woodyi* also are widely distributed in the world's oceans. Rest of the two isolates obtained from seawater at 45- and 50-m depth showed different ARDRA patterns from known luminescent bacterial species. 16S rDNA sequence analysis revealed that they had 99.8% and 99.1% similarity with *P. leiognathi* and *P. phosphoreum*, respectively. Among the band patterns of three enzymes, *Hha*I, *Rsa*I and *Eco*RI, both isolates showed restriction patterns of *Eco*RI different from the reference strains. The reasons for this phenomenon are unclear at present, although multiple copies of 16S rDNA in the genome may be involved. If the *P. leiognathi* and *P. phosphoreum* isolates possess similar variations of *Eco*RI recognition sites on 16S rDNA copies, this could create ARDRA patterns similar to the ones we found. As the ARDRA method discriminates bacterial isolates depend on their restriction patterns of 16S rRNA gene of well-described species among luminescent bacteria currently known, it may provide a unique opportunity to find luminescent bacteria that are new or phylogenetically distinct from what is currently known.

In conclusion, we developed ARDRA with five enzymes for discrimination of 14 marine luminescent bacterial species currently known. The ARDRA is rapid and useful tool for identification of natural luminescent isolates. With this method we clarified culturable luminescent bacterial diversity in a coastal area of Japan (Sagami Bay). ARDRA described here is shown

to be reliable and useful tool for the studies of ecology and diversity on luminescent bacteria in marine environments.

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B-7

Impact of the shrimp farming activity on *Vibrio* spp. diversity in Ceará State's estuaries

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Marine shrimp farming is one of the most economic attractive investment, not only in Brazil but also over the world. Farms in Northeastern Brazil account for most of the country's shrimp production. However, shrimp populations have been constantly submitted to bacterial infections caused by vibrios endemic to estuarine and freshwater environments. These bacteria are associated with the shrimp microbiota during the early larval stages and may be isolated from both healthy animals and the environment itself. The present study surveys species diversity of genus *Vibrio* occurring in four mangrove areas located on the Choró, Pirangi, Jaguaribe and Pacoti rivers estuaries, in Ceará State (Brazil), three of which with shrimp farms and one without (control). Water and sediment samples were taken inside the four estuaries as well as in the inflow and outflow channels and ponds of a *Litopenaeus vannamei* shrimp farm. 138 strains of *Vibrio* were isolated and identified from estuarine water and sediment samples and 25strains from samples colleted on the farm. The *Vibrio* species most frequently observed were: *V. parahaemolyticus*, *V. fluvialis* and *V. cholerae* non-O1 and non - O139 (in samples from the environment) and *V. cholerae* non -O1 and non- O139 (in samples from the farm).

Results suggest that, since vibrios were found in estuaries both with and without shrimp farming activities and the species diversity was greater in the environment than on the shrimp farm, estuaries are not negatively impacted by shrimp culture as far as the genus *Vibrio* is concerned.

Key words: *Vibrio*, mangrove, shrimp farming

B-8

Occurrence and Composition of Class 1 and Class 2 Integrons in Clinical and Environmental O1 and Non-O1/Non-O139 *Vibrio cholerae* strains from the Brazilian Amazon Region.

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The integrase (IntI) is the signature of an integron. So far, three classes of integrons (class 1, 2 and 3) have been described associated with resistance gene cassettes. Class 1 is recognized as the most widespread among clinical isolates. This element is characterized by a 5' conserved segment (CS), which includes the integrase gene (*intI*), the recombination site (*attI*) and a promoter region (P*ant*), and a 3' CS which usually includes the *qacEΔ1* and *sulI* genes. In between these CSs, there is a variable region where gene cassettes can be inserted and expressed under P*ant* control [1,2,3,4]. Class 2 and 3 integrons contain the integrase genes (*intI*2 and *intI*3), and are not widespread among bacteria as class 1 integrons. Class 2 integrons have been identified in association with Tn*7* transposon in *Escherichia coli, Acinetobacter baumannii* and *Shigella sonnei* strains. There are several studies showing class 1 integrons in *Vibrio cholerae* strains in European and Asian countries.

In this work we identified and characterised class 1 and class 2 integrons in clinical and environmental isolates of *V. cholerae* O1 and non-O1/non-O139 from Brazilian Amazon region . In order to verify the presence of gene cassettes within strains carrying the integrase gene (*intI*1), PCR reactions were performed using primers targeting the conserved sequences flanking the variable region. The amplicons corresponding to integrase gene, the variable region and de 3' conserved region were directly sequenced. Amplicons were purified using Wizard SV Gel and PCR Clean-UP System kit (Promega). Sequencing reactions were performed with Big Dye Terminator RR Mix (PE Apllied Biosystems) in a ABI Prism DNA Sequencer 377 (Applied Biosystems). Nucleotide sequences were compared to those available in Genbank database accessible on the National Center of Biotechnology Information web site.

The sequence analysis of the variable segment revealed the presence of two alleles of the *aad*A gene: *aad*A2 and *aad*A7 which confers resistance to aminoglycosides as streptomycin and spectinomycin . These cassettes were found in the variable regions detected in isolates of

two environmental *V. cholerae* nonO1/nonO139 clones. The isolates of clone 1 (determined by MLEE) were isolated from sewage in 1977 and carried the *aad*A2 gene. The clone 2 persisted in sewage from 1978 to 1981 and harboured the *aad*A7 gene. In clinical isolates of *V. cholerae* O1, belonging to the South America cholera epidemic clone (characterized by MLEE and PFGE), we detected the integrase gene (*intI*1), and 3' CS including the *qacEΔ1* and *sulI* genes. However, the analysis of the integron variable region revealed that the isolates were devoid of gene cassettes, indicating a high occurrence of empty class 1 integrons among them.

In the early days of cholera epidemic in Brazil, in 1991, a new lineage of *V. cholerae* O1 was identified, the Amazonia strain. Interestingly, in one clinical isolate of this lineage we determined the presence of the rare Class 2 integron. Sequence analysis showed that it was composed and organized as: *intI* 2/*sat*/*aad*A/*orf*X (resistance to streptothricin and streptomycin).

Class 1 integrons carrying resistance gene cassettes had been detected in *V. cholerae* O1 and non-O1/non-O139 in Europa and Asia and also the presence of MegaIntegron characterized by the class 4 inegrase [6,7]. The analysis indicated the presence of distinct structures among strains and heterogeneity in organization and/or composition of the variable elements [8]. However in our work, a large proportion of clinical *V. cholerae* O1 strains from Brazilian cholera epidemic, was shown to carry empty Class 1 integron. In environmental *V. cholerae* non-O1/non-O139 isolates from a period before the cholera epidemic, alleles of *aad*A gene were in the variable region of Class 1 integrons. Here, by the first time, is reported the presence of the rare class 2 integron in *V. cholerae,* and the sequence analysis determined the entire presence of the class 2 integrase.

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B-9

Phenotypic resemblance among *Vibrio alginolyticus* strains isolated in North-Western Adriatic Sea and in South-Western Atlantic Ocean

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For more than 400 years we have been facing globalization, *id est*, the free circulation of goods and persons all over the world (according to the will of economy). Together with globalization of goods carriage and human transport, a global dispersion of localized diseases has begun. This process is still occurring. Despite of the pandemics of cholera, we are now facing pandemic strains of *V.parahaemolyticus*. The recent pandemic distribution of bacteria that are so linked to environmental conditions fits well also with problems such as global warming, "El Niño" and modifications of oceanic currents flow. Recent developments in bacteriological study are making the list of potentially pathogenic species longer and longer. For each new pathogen, one question rises: is it a problem of local or global concern? Until five years ago, *Vibrio alginolyticus* was considered an opportunistic bacterium. Now it is known as a very frequent potentially dermatopathogenic species, widespread all over the world. In this study we analyzed the phenotypic resemblance among strains isolated from the northwestern Adriatic Sea (Lagoon of Venice, Italy) and from the southwestern Atlantic Ocean (Guanabara Bay, Rio de Janeiro, Brazil). The likeness was analyzed through the prevalence of potentially high pathogenic strains (possess of ellastase, chondroitinase and collagenase enzymes), pattern of antibiotic-resistance and resistance to heavy metals. This study included 33 *Vibrio alginolyticus* strains isolated from bivalve mollusks in the lagoon of Venice (Italy), and 51 strains isolated in Brazil: 27 from mussels; 24 from pelagic cetacean species. It was analyzed the antimicrobial susceptibility test performed by MIC according to CLSI- 2005

recommendations by the following antimicrobial drugs: Ampicillin, Tetracycline, Ciprofloxacin, Cefoxitin, Nitrofurantoin, Chloramphenicol, Streptomycine, Trimethoprim Sulfamethoxazole, Eritromycin, Gentamicin and Nalidixic Acid and the heavy metals: lead, copper, cadmium and mercury. For quality control, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 were tested under the same conditions for antimicrobial and heavy metals suggested by CLSI (NCCLS), 2005. Results have been statistically analyzed using "chi-square test", searching significative differences between the average result of all analyzed strains together and the average result of the analyzed clusters. No significative difference in the result of MIC test was shown between 0,5% and 1% NaCl antibiotic solutions. The required time to reach 0,5 McFarland opacity in the microwell plates showed a great variability. Most of analyzed strains required from 2 to 2 and half hours. No significative differences have been found in the prevalence of potentially high pathogenic strains. All analyzed strains were sensitive to Tetracycline, Ciprofloxacin, Nitrofurantoin, Chloramphenicol, Trimethoprim Sulfamethoxazole and Nalidixic Acid. Some strains showed resistance to Cefoxitine (4,76% always together with Ampicillin resistance). 2 strains were intermediate resistant to Gentamicin (2,38%). A high percentage of strains showed resistance to Ampicillin (85,7% of analyzed strains). Differences in prevalence of resistant strains in Italian, Brazilian and cetacean strains were not significative by 95% confidence degrees, neither from qualitative, nor quantitative point of view. From the results we have so far obtained, we can see an high phenotypic similarity between strains isolated from Adriatic Sea and SW Atlantic Ocean. By now we cannot say if these results are linked to evolutionary convergence and adaptation to highly polluted seas, or if pandemic strains of *Vibrio alginolyticus* exist.

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B-10

Partial *gyrB, toxR,* hemolysin and *lux* Gene Sequences As Alternative Markers to Differentiate *Vibrio harveyi* from *Vibrio campbellii*

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In this study, the partial *gyrB, toxR* and hemolysin genes from type strains of *V. harveyi* (NBRC 15634) *and V. campbellii* (NBRC 15631 and CAIM 519) were amplified, isolated, and sequenced resulting in the first report of *toxR* and hemolysin and *gyr B* nucleotide sequences from these species submitted to the GenBank. Multiple sequence alignment revealed that the two species exhibit percent similarity of 93-95%, 74-76% and 82-83% for *gyrB, toxR* and hemolysin genes, respectively. Moreover, the use of *lux* primers in PCR resulted in distinct and different profiles for these two species. Phylogenetic analysis was also performed based on the partial sequences of *toxR*, hemolysin genes, and *gyrB* gene sequences, suggesting that these genes are more useful in distinguishing these closely related *Vibrios* (*V. harveyi* and *V. campbellii*), compared to the 16S rRNA gene. Lastly, primers for *toxR* and hemolysin gene-targeted PCR were designed based on available sequences and tested using optimized PCR parameters, thus developing a PCR detection protocol for type strain *V. harveyi* and *V. campbellii*.

B-11

Vibrio species associated with the culture of clams in the north-west of Spain.

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Shellfish aquaculture, in particular the clam culture, is of great importance in of Galicia, (north-west of Spain). The overexplotation of natural beds have led to the import of seed and adult clams with the subsequent risk of introduction of microorganisms leading to new pathologies (i.e. the appearance of the brown ring disease). Previous studies have described the diversity

among *Vibrio* species and bacteria from other genera (*Acinetobacter* and *Pseudomonas*) in molluscs (Castro et al. 2002, Pujalte et al. 1999), but the scarce knowledge of potential bacterial pathogens for clam makes worth considering the development of further studies for their detection and characterization. In this study bacteria present in cultured clam populations (*Ruditappes philippinarum* and *R. decussatus*) of 4 different locations was analyzed over a period of 17 months and characterization of fermentative bacteria was performed. The phenotypic identification was performed by standard biochemical techniques (West and Colwell 1984). The study analysed the evolution of the total number of bacteria, as well as a characterization of the more prevalent associated species in the samples, being focused mainly in the genera *Vibrio*. A total number of 876 fermentative strains were isolated, from which 80,0% were from the genera *Vibrio*, 13,4% from the genera *Aeromonas* and the 6,6% were fermentative strains not identified. The total number of *Vibrios* isolated were 701, the most abundant species were *V. splendidus I / V. anguillarum* with 45,9%, *V. splendidus II* with 10,4%, *V. diazotrophicus* with 5,4% and *V. alginolyticus* with 6,0%. Distribution of vibrios in the four sampling locations was similar, with the exception of *V. splendidus I / V. anguillarum*, being significantly more abundant in one of the sites sampled. Two strains of the pathogen *V. tapetis* were isolated from two different sites; symptoms of the disease were also detected by histological examination. PCR detection of this pathogen by means of specific primers was performed from mixed cultures on plate media (Rodríguez et al. 2003). The pathogen was detected in a high number of samples (aprox. 50%) proving that *V. tapetis* is present in clams, but the presence of other dominant bacteria makes difficult its isolation. Detection and characterization of the bacteria obtained will determine their potential pathology on clams; the study of the virulence factors will be important to further understand the pathobiological factors determinant in the development of bacterial diseases in the adult stage of this bivalve mollusc.

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B-12

New vibrios isolated from disease outbreaks in shellfish larvae

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Shellfish aquaculture represents an economically important activity worldwide. In the case of bivalves, decline in productivity of natural beds has resulted in the need of the development of hatcheries to supply spat to farmers. But these facilities suffer losses by the high incidence of epizootics among larval and juvenile stocks, affecting growth and survivability. The role of bacteria of genus *Vibrio* in outbreaks affecting larval and post-larval stages have been widely reported. Nowadays, although a number of pathogenic species have been already identified, further studies are necessary to understand the diversity of vibrios in aquaculture systems.
The aim of this study was the identification of three *Vibrio* strains, aetiological agents in three outbreaks of disease in three different molluscan hatcheries in Galicia (NW Spain). Strains PP-145.98, PP-203 and PP-638 were selected among the isolates obtained from these episodes by their demonstrated pathogenicity in laboratory experiments carried out with flat oyster (*Ostrea edulis L.*) larvae. Selected isolates were examined for their biochemical and physiological characteristics according the standard procedures for marine bacteria, including NaCl requirement, temperature range of growth and susceptibility to several chemotherapeutant agents. Additional phenotypic analyses were performed using the API systems (API20E, API20NE and API ZYM; bioMérieux). Genetic characterization by sequencing of the 16S rRNA gene was also carried out.
Phenotypic and genetic analyses allowed us to assign them to genus *Vibrio*. One of them, PP-145.98, could be identified as *Vibrio neptunius* by sequencing of 16S rRNA gene (99.47% similarity). In the case of PP-203, the closest phylogenetic relatives, with similarities between 97.38 and 97.07%, were *V. vulnificus*, *V. aestuarianus*, *V. splendidus* biovar II, *V. parahaemolyticus* and *V. chagassi*. Strain PP-638 was more similar to *V. orientalis*, *V. campbellii*, *V. mytili* and *V. tubiashii* (96.93 - 96.51%). Therefore, strains PP-203 and PP-638 may represent new species within genus *Vibrio*, based on their characteristics and the differences with described *Vibrio* species.

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B-13

Molecular characterization of *Vibrio* isolated from shrimp hatcheries in Indonesia

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The presence of *Vibrio* in shrimp aquaculture industry is of a great concern. Firstly it is related to the disease for the shrimps, and secondly is related to food safety issue for human consumption. Luminescent vibriosis caused by *Vibrio harveyi* is one of the main bacterial diseases of penaeid shrimp. Vibriosis could result in high mortality rate and caused dramatic financial loss to the marine aquaculture industry. Shrimps are affected by vibriosis throughout different stages of life, from larvae, postlarvae, juveniles, subadults and adult animals. In shrimp hatchery operation, surveillance for *Vibrio* presence in shrimp fry is a routine practice for quality control and disease monitoring. However, the methods are limited in the detection of certain species only and can be quite tedious. Therefore, a rapid and accurate system is needed to identify potentially harmful *Vibrio* species associated with shrimp fry. For the purpose of identification of *Vibrio* at the species and strains levels, genomic analysis such as repetitive extragenic palindromic PCR (rep-PCR) was proven to be useful.

Here we report the combination of microbiology analysis and rep-PCR in characterizing *Vibrio* isolated from shrimp hatchery in Indonesia. As much as 30 Vibrio isolates collected from two different shrimp hatcheries in Sumatera, Indonesia were subjected to the analysis. ATCC type strains were employed as reference strains. Microbiological analysis was conducted through selective media for *Vibrio*, followed by rep-PCR using the (GTG)5 primer (Gomez-Gil *et al*., 2004). Molecular fingerprinting analysis by BIOLOG, and 16S-*rRNA* sequence analysis were conducted for back up confirmation. Results from rep-PCR were used for cluster analysis of the *Vibrio* isolates. In addition, correlation between cluster analysis and pathogenicity level to shrimp fry and other physiological traits will also be presented.

Gomez-Gil, B., *et al*., Microbiology. 2004, 150:1769-1777

E-1

Characterization of antibiotic resistance genes in *Vibrio cholerae* isolated from ships' ballast tanks

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Ships' ballast has been implicated as a vector for the dispersal of invasive species into new environments. Numerous cases have been documented for the introduction of macroorganisms via ballasting operations, but what is less known is the role of ballast water and residuals in the spread of potentially harmful microbes, especially with reference to novel genotypes (Ruiz et al., 2000). Of particular interest are the genes encoding for various forms of antibiotic resistance, many of which are carried on mobile genetic elements. A three-year sampling effort has yielded over 300 putative isolates of *Vibrio cholerae* from ships' ballast tanks and various environmental sources, of which 208 have been profiled for antibiotic susceptibility using twelve diverse antibiotics. The results demonstrate widespread resistance to beta-lactams (74.6%), with isolated instances of resistance to other antibiotics. Plasmid extractions and restriction enzyme analyses have shown the presence of an ostensible plasmid of approximately 60 kbp in many of these isolates. Additional work is being done using restriction fragment length polymorphisms and PCR for specific antibiotic resistance genes in an attempt to characterize these plasmids. Knowledge from this study and future microcosm experiments will help us to ascertain the potential for horizontal gene transfer in a ballast tank setting.

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E-2

Vibrio cholerae O1 and O139 in the Aquatic Environment of Mathbaria, Bangladesh

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Toxigenic V*ibrio cholerae* O1 and O139, the causative agents of epidemic and pandemic cholera, cause seasonal outbreaks in the Ganges Delta region. Despite being autochthonous to the aquatic environment, toxigenic *V. cholerae* O1 O139 are rarely isolated from the aquatic environment even during epidemic periods by conventional bacteriological culture methods. Nonetheless, toxigenic *V. cholerae* can be detected in the aquatic environment using direct detection methods, i.e. the cells are in a viable but non-culturable state, failing to grow on standard microbiological culture plates. In this study, we examined biofilms for the presence of culturable *V. cholerae* including non-epidemic periods. Biweekly environmental surveillance for *V. cholerae* was carried out in Mathbaria, a cholera endemic area adjacent to the Bay of Bengal, with the focus on *V. cholerae* O1 and O139 Bengal. A total of 297 samples of water, phytoplankton, and zooplankton were collected between March and December, 2004, yielding eight *V. cholerae* O1 and four O139 Bengal isolates. A combination of culture methods (CM), multiplex-polymerase chain reaction (M-PCR), and direct fluorescent antibody-direct viable count (DFA-DVC) revealed the Mathbaria aquatic environment to be a reservoir for *V. cholerae* O1 and O139 Bengal. DFA results showed significant clustering of *V. cholerae* in biofilms during the inter-epidemic period. Fluorescent micrographs revealed large numbers of *V. cholerae* O1 in thin films of exo-polysaccharides (biofilm). A similar clustering of *V. cholerae* O1 was also observed in biofilm samples collected from Matlab, Bangladesh. Thus, the results of this study provide *in situ* evidence for the presence year round of *V. cholerae* O1 and O139 in the aquatic environment, predominantly as viable but non-culturable cells, but also with some culturable cells in the biofilm consortia. The biofilm community is concluded to be an important factor in the incidence of cholera bacteria in the aquatic environment, especially between the seasonal epidemics of cholera in Bangladesh.

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E-3

Characterization of Cryptic Plasmids from Environmental *Vibrio* Species Isolated from Coastal Marine Environments

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Vibrio species are the natural inhabitants of aquatic environments. They live either freely in brackish water or in association with plankton. Consumption of insufficiently cooked seafood, accidental drinking of *Vibrio*-contaminated water or wound exposure to *Vibrio*-containing water is the most common means responsible for *Vibrio*-infections. The co-dwelling of *Vibrio* species with marine animals may lead to possible transmission to humans. *Vibrio* species have also been listed as one of the leading bacterial pathogens in avian diseases. *Vibrio mestchnikovii* was isolated and identified from diseased domestic ducks and geese in Germany. The enteritis in avian species like canaries and finches was caused by *Vibrio parahaemolyticus*.

Mai Po Nature Reserve is threatened by increasing pollutions largely due to the recently economic development in the adjacent Shenzhen Special Economic Zone of the People's Republic of China. Large quantities of domestic and industrial wastewaters have been discharged to the inter-tidal area without proper treatment. Water quality monitoring carried out by Hong Kong SAR Government indicated that Mai Po Nature Reserve is one of the most polluted water bodies in Hong Kong. The status of pollution has not been improved in the last several years. In contrast, Cape d'Aguilar Marine Reserve of Hong Kong is also a protected area situated in Cape d'Aguilar Peninsula, southeast tip of Hong Kong, and the environment is pristine nature and clean. Though research has been conducted on the basic biology of flora and fauna in the area, investigation on microorganisms especially opportunistic water-borne pathogens have not been conducted before.

Water samples were collected from several previously determined sampling sites at Mai Po Nature Reserve (22°29′N to 22°31′N and 113°59′E to 114°03′E) and Cape d'Aguilar Marine Reserve (22°12′N and 114°15′E) of Hong Kong. Relevant information can also be found elsewhere (Wang and Gu, 2005; Wang et al., 2004). Surface water was taken in 1 L plastic bottles when the tidal level was at approximately 1.5 m at Mai Po and Cape d'Aguilar. All the samples were transferred back to the laboratory immediately after sampling for processing. The samples used in this investigation were collected in May 2005.

Fifty environmental isolates of *Vibrio* species were isolated from water samples of Mai Po Nature Reserve and the Cape d'Aguilar Marine Reserve in Hong Kong and they were screened for the presence of plasmids. Mai Po is a wastewater-impacted area while the Cape d'Aguilar Marine Reserve is a pristine marine water. Plasmids were found in *Vibrio* isolates from both

sites at similar frequencies and each site had distinctive plasmid profiles. These plasmid-bearing *Vibrio* isolates were identified to be different species of the *Vibrio* species by both biochemical test and subsequent full-length 16S rRNA sequences. Antibiotic resistance test showed that all these plasmid-bearing *Vibrio* isolates showed multiple resistance to the 21 antibiotics tested.

All these plasmid-bearing *Vibrio* isolates showed multiple-resistance to the 21 antibiotics tested in this study. Although they belong to different species of the *Vibrio* genera, they displayed some common antibiotic resistance profile, all isolates were resistant to ampicillin, carbenicillin, cephalothin, clindamycin, colistin sulphate, erythromycin, fusidic acid, methicillin, nitrofurantoin and penicillin G. The presence of plasmid(s) in *V. cholerae*, mainly in pathogenic strains during the cholera outbreaks is not rare. *V. cholerae* O1 El Tor from Bangladesh was found to carry multiple antibiotic resistance genes. The presence of both resistance of antibiotics and the large plasmid in *Vibrio* isolates may have significant ecological and public health implications. In addition, selective isolates also showed tolerance to 10 μM Hg^{2+} in culture medium and these isolates generally harbored large plasmid(s) (>30 kb).

A novel cryptic plasmid, pVC, from an environmental isolate of *Vibrio cholerae* MP-1 from Mai Po Nature Reserve in Hong Kong has been isolated. The complete nucleotide sequence analysis (3,806 bp) revealed three major putative open reading frames (ORFs). ORF-1 encodes a putative protein of 173 amino acids with a predicted molecular weight (MW) of 19.9 kDalton (kD). The analysis of ORF-2 demonstrated that its translated product has 171 amino acids and 31% sequence homology to an ORF-II encoded by a colicigenic plasmid in *Salmonella enterica*. ORF-3, 117 amino acids in length, has a predicted MW of 13.3 kD and shows 44% similarity to a conserved hypothetical protein in metal-reducing *Geobacter sulfurreducens*. Two identical AT-rich direct repeats (AATAAATATT) were detected at nucleotides 2259-2268 and 2294-2303, Elevated temperature (42°C), high concentration of ethidium bromide (500 μg/ml) and sodium dodecyl sulfate (5%) treatments all failed to cure pVC from its host strain, suggesting a likely close relationship to the host strain over the evolutionary history.

Our results showed that high frequency of plasmid in *Vibrio* species of both polluted and pristine environments may be ecologically important to the survival of these bacteria in the environment. The specific functioning of the cryptic plasmids remains the focus of current investigations.

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E-4

Role of *Vibrio vulnificus* type IV pili in persistence in oysters, *Crassostrea virginica*

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Vibrio vulnificus is part of the natural estuarine microflora, and is especially common in sub tropical and tropical marine environments. These bacteria associate with marine microflora and can accumulate in shellfish through filter feeding. *Vibrio vulnificus* can cause fatal septicemic infections in individuals who are immunocompromised or suffer from liver disease, by consumption of raw shellfish colonized by the bacteria. In addition, the bacterium can cause severe necrotizing wound infections in otherwise healthy people who handle shellfish harboring the bacterium. Such infections pose a significant threat to human health and impact the shellfish industry causing significant economic losses. Little is known about the specific interactions of these microorganisms with shellfish that result in significant accumulation of the bacteria. Our research is aimed at the characterization of surface structures on the bacteria, specifically pili or fimbriae to ascertain their function in persistence of *V. vulnificus* in oysters, as well as their role in human pathogenesis. Studies on the role of a specific class of pili in *V. vulnificus*, designated type IV, have demonstrated their role in adherence to human epithelial cells, biofilm formation, and virulence in a mouse model. Preliminary studies indicate that these pili also contribute to persistence of the bacterium in oysters, *Crassostrea virginica*. If these factors prove to be responsible for the bacterium's ability to colonize oyster tissue, they may present a unique and specific target(s) for compounds designed to interfere with this attachment, leading to depuration methods that could potentially reduce or eliminate the organisms from oysters.

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E-5

Sensitive and rapid detection of viable *Vibrio cholerae* cells by a Nucleic Acid Sequence Based Amplification (NASBA) method in water

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This study describes the development of a highly sensitive, rapid and specific method for detection of viable *Vibrio cholerae* cells using the nucleic acid sequence based amplification (NASBA) method and molecular beacons for real-time detection. NASBA is a robust RNA selective amplification method developed during the last decade for detection of several pathogenic viruses and bacteria. Advantages of the method compared to reverse transcriptase PCR includes that the NASBA reaction is isothermal (41°C), and that DNA-free RNA is not required as template.

The objective of this study was to use NASBA for detection and identification of *V. cholerae*. Nucleic acids (RNA and DNA) were isolated from a *V. cholerae* culture grown to mid-exponential growth phase in TSB solution by the Nucliscens Basic isolation kit (OrganonTeknika) and the isolated RNA was amplified using the Nucliscens NASBA kit (OrganonTeknika).

Genes involved in the pathogenicity of *V. cholerae* are well documented. The cholera toxin gene, *ctxA*, and the toxin co-regulated pilus gene, *tcpA* (colonization factor), are present in most toxigenic classical and El tor biotypes. The ToxR protein regulates expression of *ctxA*. We have developed a highly specific and sensitive assay for rapid detection of *V. cholerae*. *V. cholerae* was identified by five different genetic markers, and the targets are the *toxR*, *ctxA*, *tcpA*, *hly*, and *groEL* genes. The *groEL* gene-product is a 60kDa chaperonin protein and *hly* encodes the virulence gene hemolysin A. For evaluation of different primers and beacons the toxigenic *El tor* strain, CIP106855, was used. Isolated nucleic acids were treated by DNase and RNase to make sure that RNA and not DNA were amplified. After RNase treatment no NASBA amplification could be detected. Different strains of *V. cholerae* have been tested (some of these were environmental strains lacking the cholera toxin genes). NASBA is a sensitive method and the results showed that less than 20 cells could be detected. The selectivity of the assay was also tested by amplification of *V. cholerae* in a complex sample of a mixture of cells from different bacterial species. Preliminary studies in which environmental water samples have been spiked with *V. cholerae* cells are also ongoing work. Our results strongly indicate that NASBA could be a useful method for detection of V. *cholerae* in its natural aquatic environment.

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E-6

Development of a MLVA analysis for genetic fingerprinting of *Vibrio cholerae*

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Vibrio cholerae is the etiological agent of cholera, a disease leading to between 100 000 and 200 000 cases a year worldwide. The disease is endemic in Latin America, Africa, and Asia, but there are more than 100 years since this disease were more prevalent in temperate regions as Europe. The last epidemic of cholera in Norway was in 1883, but between 1975 and 2002 there have not been reported more than 5 imported cases. CDC has classified this organism as a list B agent because it is quit easy to disseminate, has moderate morbidity and most probably will lead to public fear if it shows up as result of a bioterror action. Therefore it is important to have a capability to detect and identify this pathogen. We are now developing a MLVA (Multi Locus VNTR Analysis) application for genetic fingerprinting of *V. cholerae*, a method that has gained increased attention because of its simplicity and high discriminating power. The method is a PCR-based analysis of VNTR's (Variable Number of Tandem Repeats), where differences in number of tandem repeats on multiple loci on the bacterial chromosome is studied. Specific primers are developed for flanking sequences of selected tandem repeat regions. Agarose gel or different capillary sequencers are used to analyse the repeat number of the generated PCR fragments. Different isolates are defined by a MLVA profile corresponding to the number of repeats at each different VNTR loci. Cluster analysis on the MLVA profiles can then be performed and genotypic relations be presented graphically by phylogenetic trees. So far we have found several stable markers giving polymorphic fragments from a collection of both clinical and marine isolates of *V. cholerae*.

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E-7

Construction of a set of green fluorescent protein-tagged vibrios for studying host-microbe interactions

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Vibrios are important model bacteria to study host-microbe interactions involving both human and marine animal hosts. Bacteria tagged with green fluorescent protein (GFP) represent a powerful tool for investigating host-microbe interactions of vibrios. However, a *Escherichia coli* conjugal transfer system of broad-host-range plasmids harboring a *gfp* gene has not been fully applicable to all vibrios, because most vibrio recipient strains were not outgrown by commonly used *gfp*-donor *E. coli* in a standard selective condition. We evaluated effective outgrowing conditions of vibrios as *gfp* recipients, and refined conjugative effectiveness to make GFP-tagged vibrios. Specifically, we found that vibrios transconjugants outgrew the *E. coli* donor when mating mixes were plated on an alginate-containing marine agar at 15°C, making this a suitable selective condition. Forty-three strains of GFP-tagged vibrios were constructed using this system. Moreover, we characterized the GFP-expressing cell ratio under aerobic and anaerobic culture conditions, and the decaying ratio of *gfp*-fluorescing cells under a nutrient poor condition in three species of representative GFP-tagged vibrios. The percentage of GFP expressing cells in GFP-tagged *V. halioticoli* IAM14596^T, *V. pelagius* ATCC 25916^T, and *V. alginolyticus* LMG 4409^T was higher at 15 °C (49-54% of cells were fluorescent) than at 20- or 25 °C-culture. The GFP-expressing cell ratios were higher during aerobic rather than anaerobic culture (33-38% of cells were fluorescent). Furthermore, these GFP-expressing cells of *V. halioticoli*, *V. pelagius*, and *V. alginolyticus* are detectable within 4 days in nutrient poor artificial seawater at 15 to 25°C.

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E-8

Adaptive features in cold water V*ibrio* pathogens

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Farming of fish in cold marine environments has resulted in occurrence of "new" infectious diseases caused by cold water *Vibrio* species. *Vibrio salmonicida* is causing cold water vibriosis in farmed Atlantic salmon (*Salmo salar*) and farmed cod (*Gadhus morhua*) and to some extent in rainbow trout (*Salmo gardneri*) farmed in marine water. The bacterium caused considerable loss in Atlantic salmon farms in Norway, Scotland, Iceland and in the Bay of Fundy area in Maine, USA and New Brunswick, Canada in the 1980's. Vaccination and improved fish farm management have effectively controlled the disease the last 15 years.
Moritella viscosa (formerly *Vibrio viscosus*) is causing winter ulcer in farmed Atlantic salmon and farmed cod. Winter ulcer occurs when the water temperature is below 7 – 8°C. The disease has been observed in Norway, Iceland and the Shetland Islands since the beginning of the 1980's. Winter ulcer is the most common bacterial infection in the farming of Atlantic salmon in Northern Europe today. Vaccination is not always effective and to some extent disease outbreaks have to be controlled by antibiotics.
Vibrio wodanis is a psychrophilic *Vibrio* isolated together with *M. viscosa* or as the only bacterium from ulcers and from kidneys in up to 60 % of fish with winter ulcer. In challenge experiments, however, *V. wodanis* has not caused winter ulcer.
Phenotypical studies of *M. viscosa* and *V. salmonicida* show that these pathogens respond to changes in the environment in a similar manner. The ability to adhere to the surface of agar plates increase with low incubation temperatures. Similarily, adhesion to the agar surface increases with lower concentration of sodium chloride in the medium. On the contrary, low concentrations of sodium chloride reduce the motility of the bacterial cells tested as ability to swarm on the surface of semi-solid agar.
Both bacteria are able to adapt to the physico-chemical conditions in the fish tissues when winter ulcer and cold water vibriosis is developing in the host. Increased ability to adhere to the cells of the tissue surface and reduced need of motility within the infected tissues compared to the marine water are important adaptive features. The reasons for activation of factors important in pathogenicity of *M. viscosa* and *V. salmonicida* at cold temperatures only are not known.
Two-dimensional gel studies of proteins expressed by *M. viscosa* at different temperatures and sodium chloride concentrations reveal that only a few proteins vary according to these parameters. The studies will contribute to an increased knowledge of the behaviour of cold water vibrios.

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E-9

TCA cycle components as the resuscitation factors of *Vibrio parahaemolyticus* from the VBNC state

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The viable but nonculturable (VBNC) state is one of survival strategies for many bacteria (incl. pathogenic and non-pathogenic) when they are exposed to environmental stress. The physiological and molecular basis for the VBNC state of bacteria have not been fully elucidated though there are many reports on the environmental factors inducing the VBNC state and the resuscitation factors from the VBNC state by using various kinds of bacterial species.

Among diverse environmental stresses, low nutrient concentration at low temperature is the most widely used to induce the bacterial VBNC state in many laboratories. Pyruvate and catalase supplied onto the conventional growth media are known as the most effective agent for the resuscitation from the VBNC state of various bacteria (1). Reaction with H2O2 and the elimination of the toxicity of the reactive oxygen species (ROS) is the only explanation of their resuscitation mechanisms. Furthermore the possibilities of *in vivo* resuscitation in the animal or human gastrointestinal tract are not definitively characterized yet (2).

On our poster presentation the various conditions to induce VBNC state of *Vibrio parahaemolyticus* will be shown including nutrient abundant media such as PBS, Alkaline Peptone and Salt Polymyxin Broth (each sodium chloride's concentration was 3%) and several temperatures (2-20 degrees C). As for the safety of the food which is generally rich in nutrients and in natural bacterial populations it would be very important to elucidate the possibility of the food pathogen to enter into the VBNC state during processing or preservation and also to establish the effective, inexpensive, and simple detection system of them. Our results show that *V. parahaemolyticus* could easily enter into the VBNC state in rich growth media such as Alkaline Peptone and Salt Polymyxin Broth at low temperature and the resuscitation ability was maintained over a long preservation period.

In our trials to find resuscitation factors of *V. parahaemolyticus* in the gastrointestinal environment, we isolated a bacterium, identified as *Staphylococcus lentus* from a rat's fecal extracts. The culture broth of *S. lentus* added with 50% showed the highest resuscitation activity on nutrient plate agar though the resuscitation ratio was not as high as those of pyruvate or catalase. The mechanism of this resuscitation activity was discussed: pyruvate and catalase could resuscitate from the VBNC state of *V. parahaemolyticus*, corresponding to the previous

reports. The scavenging activity of H2O2 were observed with pyruvate in the nutrient broth as well as catalase, however, the activity was not correlated with the resuscitation ratio. Indeed each scavenging activity was almost the same despite pyruvate's resuscitation ratio was 10-fold higher than catalase's.

This observation led us to investigate the other resuscitation mechanism of pyruvate: TCA cycle plays an important role in generating energy and synthesizing various cellular components and pyruvate is the central molecule of this cycle. Metabolic contribution of pyruvate to resuscitate the VBNC state attracted less attention. The compounds related to the TCA cycle such as citric acid, 2-oxoglutaric acid, succinate, fumarate, malate, oxalacetic acid and glyoxylic acid (each concentration was 0.1%/plate) were tested on the resuscitation effects and H2O2 scavenging activities. As a result there were scavenging activities without succinate and fumarate. Resuscitation effect was observed with oxalacetic acid, 2-oxoglutaric acid in TCA cycle and glyoxylic acid whose activities were same as pyruvate. However there was no relationship between the H2O2 scavenging activity and the resuscitation ratio. The ATP contents of the VBNC state's cells before and after resuscitation treatments, which incubated VBNC state's bacteria with or without resuscitated components, were also measured but there were no differences before and after concerning the ATP volume. The role of the TCA cycle in the resuscitation process will be discussed on the poster.

In our laboratory we sometimes experienced the difficulties in obtaining the reproducible results on the VBNC preparation or resuscitation tests. The examination of the pre-culture method of the cells, cell concentration, etc. indicated that there were several unknown factors influencing the entry into the VBNC state or resuscitation ratio including the quorum sensing mechanism.

(1) Yoshimitsu Mizunoe, Sun Nyunt Wai, Takahiro Ishikawa, Akemi Takade, Shin-ichi Yoshida (2000) Resuscitation of viable but nonculturable cells of *Vibrio parahaemolyticus* at low temperature under starvation

(2) Rita R. Colwell and D. Jay Grimes (2000) Nonculturable Microorganisms in the Environment

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E-10

Population dynamics of V. *cholerae* in an inland catchment in South Africa

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Vibrio cholerae is autochthonous to aquatic environments, which serve as a possible reservoir for both enterotoxigenic *V. cholerae*, and non-enterotoxigenic environmental strains. Using non-enterotoxigenic strains as a model the possible persistence and survival of *V. cholerae* in an inland freshwater system was investigated. The genetic diversity of 100 environmental *Vibrio cholerae* strains isolated over a two-year period from the Vaal Barrage catchment, South Africa was determined using AFLP typing of the isolates. Restriction enzymes *MseI* and *EcoRI* were used to generate fragments from whole genomic DNA extracts and selective amplification was performed with the Eco-G and Mse-T primers. Similarity analysis with Pearsons correlation coefficient yielded a number of clusters, with environmental isolates grouping in various clusters, while the four clinical isolates included in the study grouped separately, together with two environmental strains. Environmental isolates grouped in clusters showing no or little resemblance to their isolation site and or isolation date. With the high level of genetic diversity seen in this study the *Vibrio cholerae* population in the Vaal Barrage system is probably not a product of one or two strains that have adapted to local conditions, but is rather made up of highly diverse clones that constantly compete, resulting in genetic shifts only perceivable within short time periods. Using environmental *Vibrio cholerae* as a model, it can be suggested that enterotoxigenic strains may exhibit a similar degree of persistence and survival in inland aquatic systems.

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E-11

Enviromental Factors Influencing *Vibrio* species population Dynamics

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To assess the environmental factors influencing Vibrio sp. population dynamics in the Neuse River Estuary (NRE) in North Carolina, water samples were collected bimonthly, and after major storm events for 15 months. Vibrio sp. were quantified by plating on thiosulfate citrate bile salts sucrose agar (TCBS) and assessed to species level using quantitative PCR. A Taqman assay targeting the vvh gene was used to detect Vibrio vulnificus from the environmental samples. To improve the detection limit of V. vulnificus a new Scorpion chemistry based assay was developed targeting the vvh gene. In situ measurements of salinity, temperature, pH, chlorophyll, turbidity, dissolved oxygen, and dissolved organic carbon were taken using a YSI 6600 (Yellow Springs Instruments Inc). In addition, particle characterization of samples was conducted using a Multisizer III coulter counter and particles collected on 0.7 mm glass fiber filters were used for particulate organic carbon (POC) measurements using a CHN Analyzer (Perkin Elmer 2400 Series II). Particle attachment of Vibrio was assessed by using a centrifugation technique to separate water samples into heavy and light particle fractions.

Over this 15 month period Vibrio colony counts showed strong geographic and seasonal variations which correlate primarily with salinity and temperature, supporting previous findings. Particle suspensions were principally composed of phytoplankton and relationships between Vibrio concentration, particulate organic carbon, and chlorophyll suggest that bacterial and algal responses to organic matter inputs were decoupled, with bacterial populations changing later and further downstream. The fraction of Vibrio cells attached to phytoplankton decreased with increasing Vibrio concentration, consistent with attachment providing a refuge for cells under unfavorable conditions. These early observations of Vibrio dynamics in the NRE, provide evidence that populations respond to organic matter inputs and interact with phytoplankton populations in addition to being influenced by salinity and temperature. Design and optimization of a Scorpion primer probe set for vvh has provided a more sensitive and rapid assessment of V. vulnificus compared to an existing Taqman design. This information may help aid assessment of pathogen concentrations in surface waters under varying environmental conditions.

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E-12

Survival of *Vibrio anguillarum*, a fish pathogen, in freshwater by forming biofilms.

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Vibriosis caused by *Vibrio anguillarum* seriously injures freshwater fish (Salmoniforms) almost every year in Lake Biwa, Japan. The pathogen needs NaCl for its life. When the pathogen was directly exposed to sterilized natural freshwater water at room temperature, it suddenly lost its culturability and pathogenicity, and died within half a day due to the low osmolarity. In this report, the survival of the pathogen as biofilms formed on air-solid and liquid-solid (agar or polystyrene) interfaces in the lfreshwater was investigated. When the biofilms formed at air-solid and liquid-solid (agar or polystyrene) interfaces were exposed to the freshwater at 4-5 C in the dark, the pathogen survived for more than 2 and 4 weeks, respectively. The biofilms at both interfaces at 4-5C in the dark enhanced the production of a mucous polymer matrix. The main constituent of the polymer was exopolysaccharide. The polymer was produced only in the dark at low temperature. At 20 C, there was no production of the polymer and the survival of the pathogen was shortened. The biofilm seemed to provide a functional consortium to support the survival of *V. anguillarum* in freshwater.

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E-13

Cloning and transcriptional regulation of Na+-NQR gene of *Vibrio anguillarum*

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Vibrio anguillarum is a moderately halophilic marine bacterium. This bacterium kills various kinds of fish over salinities ranging from seawater to freshwater, and gives serious damages to aquaculture systems. *V. anguillarum* does not necessarily require Na^+ for

growth in the presence of other seawater-cations. However, it does require Na^+ under starved conditions such as a stationary phase. It is reported that one reason why marine and/or pathogenic bacteria require Na^+ is their possession of respiration-dependent primary Na^+ pump (Na^+-NADH:quinone oxidoreductase, Na^+-NQR). In this study, we investigated the transcriptional regulation of the Na^+-NQR gene in *V. anguillarum* under various physiological conditions from logarithmic to stationary phases. The Na^+-NQR gene was cloned and its 7 kbp sequences were identified (Accession No. AB159077). Na^+-NQR of *V. anguillarum* composed of 6 open reading frames. These amino acid sequences showed homology of over 80% in comparison with those of Vibrios. S1 mapping performed against the upstream region of the nqrA gene revealed the existence of two promoters, P1 and P2. The nqrP1 was constitutively activated throughout logarithmic to stationary phase and possessed -10 (TAGACT) and -35 (ATGGCA) sequences which are similar to the consensus sequence of *Escherichia coli*. On the other hand, the nqrP2 was activated only at a stationary phase. Only the -10 (CATACT) and not -35 existed for the nqrP2. These results indicate a possibility that the nqrP2, which works specifically in stationary phase, contributes to starvation-survival of *V. anguillarum*.

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E-14

Vibrio cholerae O1 grows well with natural assimilable organic carbon (AOC) in different types of freshwater

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Growth of *Vibrio cholerae* O1 Eltor serotype Ogawa and *Salmonella typhimurium* ATCC 14028 was studied with an AOC regrowth assay where autoclaved and filtered (0.22 µm) water was inoculated with low cell concentrations (5×10^3 cells/mL) and growth was followed with selective staining and flow cytometry. *V. cholerae* showed good growth (up to 2×10^6 cells/mL) in river water, lake water and the effluent of a wastewater treatment plant. In these water samples, the AOC concentrations ranged from 60 µg/L up to 800 µg/L, and the results suggest that AOC was one of the key parameters governing growth. The maximum specific growth rates (μ_{max}) of *V. cholerae* growing on lake water at different temperatures (20, 25, 30 °C) were 0.23 h^{-1}, 0.32 h^{-1}, 0.39 h^{-1} respectively. The μ_{max} increased with higher temperature, but was always half of the μ_{max} of a similarly growing consortium of natural bacteria, isolated from the sampling site. Concurrently, in a direct batch competition experiment between *V. cholerae* and the natural consortium at different temperatures, *V. cholerae* grew

up to 10 % of the total population in each case but no significant effect of temperature was observed on the outcome of the competition. *S. typhimurium* was not able to grow in any environmental sample. Similarily, laboratory test showed that it was possible to physiologically "activate" *V. cholerae* (defined as a significant increase of ATP after two days) at a minimal glucose concentration of 10 µg/L whereas *S. typhimurium* showed "activation" only at a minimal glucose concentration of 100 µg/L. Our results conclude that *V. cholerae* is not only able to survive, but also able to grow in freshwater samples up to significant concentrations and hence to compete successfully with the natural microbial flora for AOC.

Genomics

G-1

Analysis of *lux* Box Nucleotides Required for Activity of the *Vibrio fischeri* LuxR Protein

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Acyl-homoserine lactone (AHSL) quorum sensing was discovered in the marine luminescent bacterium *Vibrio fischeri*. Quorum sensing and response allows bacteria to control gene expression in a population density dependent manner. *Vibrio fischeri* produces the AHSL N-(3-oxohexanoyl) homoserine lactone (3OC6-HSL). When this diffusible signal reaches a critical concentration it can bind to a transcription factor, which functions to activate transcription of the luminescence genes. The AHSL is produced by a synthase called LuxI and the transcription factor is LuxR. In the presence of signal LuxR binds to an inverted repeat element centered at –42.5 from the transcription start of the luminescence operon. This element is called the *lux* box. Several unlinked genes, *qsrP*, *ribB*, *acfA*, *qsrV*, and *qsr7,* are also LuxR dependent. There are now many examples of AHSL-LuxR homolog dependent gene expression systems in many other genera of Proteobacteria.

We are interested in the relationship between the *lux* box sequence and its ability to serve as a LuxR binding site. Previous work has shown that the first two nucleotides of the *lux* box are not required for LuxR binding. However, no systematic analysis of the relationship between *lux* box sequence and LuxR binding has been performed. Therefore, the aims of this work were: (i) to determine the nucleotides in the *lux* box that are required for LuxR binding and activity, (ii) use these results to construct a *lux* box consensus sequence that could be used to search the genome for possible LuxR binding sites and (iii) to determine genes that are quorum sensing regulated by transcriptome analysis.

To dissect the *lux* box we employed site-directed mutagenesis to generate mutants in every position in the *lux* box. Luminescence gene promoters with *lux* box mutations were cloned upstream of a promoterless *gfp* on a plasmid, which was introduced into *E. coli* containing a second *luxR* plasmid. The fluorescence was measured in the presence of the AHSL signal. We found that single base changes in positions 4, 5, 16, 17, 18 and 20 partially inactivated the *lux* box. Mutations in position 4 reduced activity to about 30 (T4G) and, 40 %

(T4A) of wild type levels. Mutations in position 5 had even greater effects, reducing activity to 0, 10 and 50 % of wild type. Position 16 (C) showed reduced activity only when changed to an A or G (about 5 and 40 % of wild type, respectively). Mutations in position 17 (A) showed reduction in activity only when this base was changed to a C (36 %) or a T (42 %). Position 18 seems to be critical and showed greatly reduced activity when mutated (G18A, 0 %; G18C, 15 %). Position 20 (T), on the other hand, showed reduction of activity only when mutated to an A (12 %). Other positions did not seem critical for *lux* box activity. To distinguish between positions in which the nucleotide present was critical and those that were affected because of the lack of dyad symmetry, double mutations were constructed and analyzed. For positions 4, 5, 16 and 17 restoring the palindrome with a second mutation did not restore activity. As an additional way of confirming that positions 4, 5, 16 and 17 are critical for *lux* box activity, we compared 20 *lux* box sequences from different strains and genes and looked for conserved positions. As expected, positions 4, 5, 16 and 17 were conserved among all the sequences. Other than those positions, only position 10 was conserved in all the *lux* boxes. We also constructed triple and quadruple mutations in positions 3, 4, 17 and 18 and the great majority of these showed no activity. From our mutational analysis we have constructed a minimal *lux* box consensus sequence (NNNYRNNNNNNNNNNYRKNB) and searched the *V. fischeri* genome for putative binding sites by using DNA-pattern (http://rsat.ulb.ac.be/rsat/). We identified 79 putative LuxR binding sites that are in the putative promoter regions. Because we used a minimal consensus we expect many of these sites will not function as LuxR binding sites but we also predict that LuxR binding sites should have been identified as members of this group. This approach to identification of quorum controlled genes at a global level may have general utility.

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G-2

Proteome analysis of the fish pathogen *Vibrio salmonicida* by 2-D gel electrophoresis and mass spectrometry

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Vibrio salmonicida is the causative agent of cold-water vibriosis in farmed marine fish. Due to its property as a psychrophilic organism having two different life cycles, in the environment and in the fish body, its genome is expected to harbour an array of genes encoding proteins of great diversity. To understand the behaviour of *V. salmonicida* in the different environments, functional studies have been initiated. Simultaneously, a project to sequence the whole genome of the organism is finishing.

Functional studies are carried out as proteome analysis combining two-dimensional gel electrophoresis and mass spectrometry. The image analysis; scanning of Sypro Ruby or Coomassie Blue stained gels, and alignment and matching between spots, is achieved by a combination of visual inspection and use of the PDQuest v6 software (Bio-Rad). Protein spots are manually picked using a picker head (Amersham Bioscience), and hydrolysed by trypsin. The Peptide mixture is analysed on a Q-TOF UltimaGlobal™ mass spectrometer with a nanospray ion source interface (Micromass, Manchester, UK). The system is equipped with an on-line CapLC autosampler (Micromass/Waters, Milford, MA, US) arranged with a stream select module attached directly to the nanoflow interface.

Mass spectral data are recorded by means of an automated data-dependent switching function by switching between MS and MS/MS mode based upon ion intensity, mass and charge state.

Protein identification is achieved using pkl files obtained by processing MS/MS spectral data by use of ProteinLynx Global Server 2.1 software (Micromass). Peak list data are searched against the public available NCBI non-redundant protein database, and against our local *V. salmonicida* protein database using an in house Mascot search engine (Matrix Science, London, UK). Where annotation by MAscot fails, good quality MS/MS spectra are subjected to automated *de novo* sequencing using the Peaks Studio v3.0 software (Bioinformatics Solutions, Waterloo, ON, Canada).

The whole genome sequence project is carried out following a combination of two strategies: an ordered-clone approach using a bacterial artificial chromosome (BAC) library and a whole genome shotgun strategy. From the BAC library, selected clones have been sequenced to eight times coverage. Finishing the sequencing project is achieved trough random sequencing of whole genomic shotgun libraries. The obtained sequence information is continuously assembled and annotated. The growing *V. salmonicida* genomic database is utilised for the protein identification based on MS spectra.

Reproducible phenotypic differences have been revealed between *V. salmonicida* cells grown *in vitro* in the presence and absence of oxidative stress inducers, fish skin mucus, and other factors that are considered important in the process of host invasion, multiplication and tissue colonization. This analysis is the starting point for in-depth understanding of *V. salmonicida* behaviour in different environments.

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G-3

Natural Competence and Variation in *Vibrio cholerae* Genomes: Implications for Function and Evolution

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The ability of *Vibrio cholerae* to thrive under diverse conditions ranging from estuaries to the human gut implies a genome that is multifaceted and plastic. Indeed, both of these characteristics have been described (Heidelberg, et al., 2000; Comstock et al., 1996). The *V. cholerae* genome is littered with well-documented examples of horizontal gene transfer, many of which have been mediated by phage, transposons and other specialized mechanisms for DNA mobilization.

We have recently observed (Meibom, et al., submitted) that *V. cholerae* becomes naturally competent when grown on chitin or using chitin oligosaccharides as the sole carbon source. Transformation occurs at frequencies as high as 10^{-4} and depends upon a type IV pilus induced via a still-obscure signaling cascade. Incorporation of the new DNA into the chromosome requires homology and appears to proceed via homologous recombination. Like laboratory strains derived from clinical isolates, environmental isolates from central California estuarine waters are transformable when grown on chitin.

Transformation may be an important mechanism for generating diversity within *V. cholerae* populations. To characterize the genomic diversity of and predict functional differences within *V. cholerae* isolates from a geographically limited population, we used microarray comparative genomic hybridization (mCGH). Our goal in combining the high resolution mCGH technique with a dense sampling of a local population of *V. cholerae* is to explore the fate of functionally defined regions of the *V. cholerae* genome on an ecological scale in which we can detect and resolve ongoing selection and horizontal genetic exchange.

Genomic DNA from a collection of isolates was hybridized to an amplicon microarray designed based on the published N16961 genome. Under our hybridization conditions, we detect sequences from *V. cholerae* and not sequences from other *Vibrios*. We detected both sequences missing from all of our isolates and sequences that showed variable representation between isolates. By sequencing regions of the isolate chromosomes that showed variability in the mCGH analysis, we have show that most variability is due to insertion or deletion of whole ORFs, which could have been mediated by transformation and homologous recombination. We show that the mCGH analysis can be used to predict the ability of isolates to perform biochemical functions, such as growth on different carbon sources.

Perhaps the most intriguing finding from our mCGH analysis is that we can detect single gene differences in the genomes of culturable *V. cholerae* isolated in different seasons. By correlating our mCGH data with physical and chemical analysis of the waters from which

the isolates were collected, we are working to build a model of genetic and functional differences determining the ability of different *V. cholerae* genotypes to grow in different niches.

Comstock et al., *Mol. Microbiol.* 19:815-26, 1996
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G-4

High genome plasticity within *V. vulnificus* is revealed by molecular analysis of genomic islands among 28 isolates.

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Vibrio vulnificus is a ubiquitous halophilic aquatic inhabitant. *V. vulnificus* grows best in warm water temperatures over 20°C, and is rarely found in water <17°C. *V. vulnificus* is found in association with various filter feeders such as oysters, crabs and mussels. Consumption of raw contaminated seafood or contamination of wounds with *V.vulnificus,* can lead to fatal primary septicaemia or necrotising wound infections, which are characterized by extremely rapid replication of bacteria causing extensive host tissue damage. *V.vulnificus* septicaemia is the leading cause of reported death from seafood consumption in the United States. Mortality rates in susceptible individuals can be as high as 75%. *V. vulnificus* is divided into three biotypes based on their different biochemical and biological properties. *V. vulnificus* biotype 1 strains are most frequently associated with human infections; biotype 2 strains are pathogenic to eels and occasionally humans; and biotype 3 strains are recently recovered isolates from Israel associated with people who handle St Peter's fish. Genotyping studies however, indicate that *V. vulnificus* isolates are highly variable. In addition, an understanding of the main virulence factors involved in their rapid replication and host tissue destruction is still limited. Here we examine a total of 28 *V. vulnificus* clinical and environmental isolates for the presence of 9 genomic islands (GEIs) uncovered in the sequenced strain YJ016. The 28 *V. vulnificus* isolates originated from Taiwan, Japan, Israel, the United States, Spain, France and Denmark encompassing the three biotypes. The 9 GEIs in *V. vulnificus* were identified using three methods; 1) comparative genome analysis of *V. vulnificus* strains YJ016 and CMCP6, using the Artemis Comparison Tool v3 (ACT) from the Sanger Institute http://www/sanger.ac.us/Software/ACT/, 2) Basic Local Alignment Search Tool (Blast) analysis at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/COG/) of genes upstream and downstream of tRNA, transposases and integrases, 3) identification of aberrant GC% and dinucleotide frequency of the GEIs using delta-rho WEB http://deltarho.amc.uva.nl. The 9 GEIs comprised 357 kb ranging in size from 14 kb to 117 kb. PCR and southern blot analyses

using primer pairs and DNA probes specific to each of the 9 GEIs was carried out to investigate the presence of the GEIs in the 28 natural *V. vulnifcius* isolates. One island, the 37 kb *Vibrio vulnificus* Island II designated (VVI-II) encompassing ORFs VV0130-VV0165, was present only amongst the clinical isolates examined and encoded a phage-like integrase, a multidrug resistance efflux pump, as well as a hydrolase and a lysase. Three of the GEIs designated (VVI)-III, VV-V and VV-IX are present only in strain YJ016. A truncated version of VVI-VII was revealed by ACT analysis to be present in CMCP6, and PCR analysis confirmed the presence of this region in all the natural *V. vulnificus* isolates examined. This region in CMCP6 was named VVI-3. Five of the GEIs were sporadically present in both the clinical and environmental isolates. The islands encode a number of transport systems and sugar metabolism genes as well as a number of possible virulence factors including hydrolase. The evolutionary history of the 28 isolates is determined by reconstructing gene trees based on three housekeeping gene: malate dehydrogenase and the phylogenetic distribution of the GEIs is examined.

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G-5

Identification of genomic islands unique to Pandemic *V. parahaemolyticus* 03:K6 isolates

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Vibrio parahaemolyticus is a halophilic gram-negative bacterium that is widely distributed in coastal waters woldwide. *V. parahaemolyticus* is a seafood borne pathogen, which is a major causative agent of gastroenteritis. Symptoms may be severe and include nausea, diarrhea and in some individuals abdominal cramps and fever. In recent years outbreaks of *V. parahaemolyticus* infection have increased in the United States. In Taiwan, Japan, and South east Asian countires *V. parahaemolyticus* cause over half of all food poisoning outbreaks. The appearance in 1996 of the first pandemic O3:K6 strains and their rapid worldwide spread have added impetus to understanding the molecular mechanism of pathogenesis of this organism. Similar to *V. cholerae,* most strains of *V. parahaemolyticus* are not pathogenic to humans. Clinical isolates of *V. parahaemolyticus* produce two major virulence factors; the thermostable direct haemolysin (TDH) encoded by *tdh*, classified as Kanagawa phenomenon positive strains and TDH-related haemolysin encoded by *trh*. Pandemic *V. parahaemolyticus* O3:K6 isolates all contain the *tdh* gene but not the *trh* gene. The complete genome sequence of *V. parahaemolyticus* strain RIMD2210633, a 1996 pandemic, serotype O3:K6 isolate from Japan, is available. The *V. parahaemolyticus* genome is comprised of a 3.29 Mb chromosome 1 and a 1.88 Mb chromosome 2, and contains 4,832 genes, 40% of which were annotated as hypothetical proteins. From the genome sequence two type three secretions systems (TTSS)

were identified, one on each of the chromosomes. Using bioinformatic analyses to examine aberrant GC content and codon usage bias of sequences flanking tRNA, transposase and integrase genes in the *V. parahaemolyticus* genome, we identified seven genomic island (GEI) regions. The seven GEIs identified ranged in size from 16 kb to ~100 kb and encode restriction transporters, type 1 restriction endonuclease, haemaggluttins, lipase, hydrolases, ribonulcease H1, type three secretions systems, colicins, cytotoxin integrase as well as a number of transporters. Six of the *V. parahaemolyticus* GEIs are absent from congeneric bacteria (*V. cholerae; V. vulnificus and V. fischeri*). We examined 32 *V. parahaemolyticus* isolates recovered over the last 25 years encompassing 7 serotypes and isolated in 9 countries. Seven of the *V. parahaemolyticus* isolates examined were recovered pre-1996, and none of these isolates contained six of the GEIs identified. PCR assays using primers pairs designed from each of the seven GEIs demonstrated the presence of each of the seven GEIs predominantly among pandemic O3:K6 isolates suggesting that these regions may be involved in fitness of the pandemic O3:K6 clone.

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G-6

Suppression Subtractive Hybridization (SSH) to explore strains specific genes of *Vibrio splendidus* related strains

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Bacterial genomes can show a large degree of variability, both in content and organization, not only between related genera and species but even between different strains of the same species. Indeed, different isolates from the same bacterial species have been observed to show differences of as much as 20 % in their gene content and arrangement. In addition to internal reshuffling through recombination (be it illegitimate or homologous between repeated sequences such as IS elements) lateral gene transfer (LGT) plays an indisputable role in overall genome plasticity. The importance of LGT in shaping bacterial genomes has become increasingly obvious over the past 10 years. This is largely, but not entirely, due to the rapid accumulation of the complete sequences of more than 200 prokaryotic genomes. However, in spite of the number and diversity of the genetic elements responsible (phage, genomic islands, transposons, insertion sequences, integrons....) studies have largely been confined to assembling a catalogue rather than investigating their impact on genome dynamics and evolution. Indeed these aspects are extremely poorly understood and the behaviour of only a very limited number of elements composing the horizontal gene pool has been analysed in any detail.
Vibrio splendidus is a dominant culturable *Vibrio* in coastal marine sediments, seawater and bivalves, namely oysters. The organism has long been considered as an environmental organism without any pathogenic significance, however for several years, different strains phenotypically related to this species have been associated with mortalities mainly in molluscs but also shrimps,

gorgonians and fish (for review see Le Roux and Austin, 2005). The present uncertain status of *V. splendidus* seems to be attributed to a lack of pertinent diagnostic tools for its identification and to an opportunistic statue, which considers the host immune capacity as co-factor in disease development. We have sequenced the genome of *Vibrio splendidus* in order to i) allow a broader analysis of the *Vibrio* genome plasticity and ii) have a complete access to the pathogenesis repertoire of this species. The *V. splendidus* strain that we have chosen is an oyster (*Crasssotrea gigas*) pathogen, associated to « summer mortalities »syndrome, which has been responsible for high mortality rates in oyster beds in France since 1991.
In parallel to the LGP32 genome sequencing, an approach based on subtractive hybridization enrichment between the sequenced strain and an avirulent *V. splendidus* isolate (LMG20012T) has been conducted. A total of 500 fragments, which are specific of the virulent strain genome have been isolated. They are now sequenced and their content will be characterized both in silico and in vivo, through gene disruption strategies. These data will allow us to establish the list of the LGP32 specific genes implicated in its specific virulence traits and to explore the origin and the mechanisms sustaining these gene acquisitions.

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G-7

Genetic Diversity of Super-Integron in various *Vibrio cholerae* strains.

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Integrons constitute a system for gene capture and expression composed of three key elements such as 1) a gene coding for an integrase, 2) a primary recombination site, and 3) a strong promoter. This system plays an important role in the acquisition and spread of antibiotic resistance genes among bacteria. A second type of integron initially termed as class 4 integron but now termed as super-integron, has been recently characterized in *V. cholerae* O1 strain and later among the strains belonging to the family *Vibrioneceae* as well. The *V. cholerae*

super-integron contains 179 gene cassettes in 126 kb region which corresponding to 3% of the genome. Sequencing of these cassettes from different *Vibrio* species indicate that they code for different types of adaptive functions, beyond pathogenicity and antibiotic resistance. The recent finding of super-integron structures in several bacterial species has expanded their role in genome evolution. Although genetic diversity of super-integron among several species in *Vibrionaceae* has been analyzed, little is known about the genetic diversity of super-integron among *V. cholerae* strains.

In this study, we attempted to characterize the genetic diversity of super-integron among *V. cholerae* strains (O1 classical, O1 El Tor, O139 and non-O1/non-O139) isolated from various places at different time. Eleven sets of LA-PCR primers, which can amplify the 126-kb region, were designed to examine whether the region was conserved in various *V. cholerae* strains. Moreover, one hundred and nineteen DNA porbes were prepared, which were selected from 179 gene cassettes, to examine if the genes present in super-integron in *V. cholerae* N16961 were also detected in other strains of *V. cholerae*. Analysis by LA-PCR revealed that none of the *V. cholerae* strains analyzed in this study gave all 11 amplicons with same size obtained from that of *V. cholerae* O1 strain N16961. In O1 El Tor strains, about one third of the region was not amplified. In some cases, sizes of the amplicons were either smaller or larger. In O1 classical strains examined, one strain did not give amplicons of one fifth of the region and another strain failed to give amplicon for most of the region. Two O139 strains analyzed failed to amplify half of the regions. Interestingly, most of the non-O1/non-O139 strains tested in this study did not produce right size of amplicons. Colony hybridization also suggested presence or absence of the genes located in the super-integron region in various strains of *V. cholerae* and the results were well correlated with those obtained from PCR scanning. High variation in the super-integron region of various *V. cholerae* strains may affect the virulence, antibitotic resistance or adaptation to the environment of *V. cholerae* strain which needs further investigation.

G-8

Molecular evolutionary analysis of the sialic acid metabolism gene cluster (*nan-nag*) among bacteria

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Vibrio cholerae is a natural inhabitant of brackish and estuarine water, although it is more infamously known to be the etiological agent of the severe diarrhoeal disease cholera. Only strains belonging to the serogroups O1 and O139 are associated with epidemic and pandemic cholera. Several genes are found to be involved in the virulence of these strains, the *ctxAB* genes, which are part of a filamentous phage (CTX˜) and are responsible for the production of cholera toxin (CT), are the main cause of the profuse watery diarrhoea characteristic of

cholera. The toxin co-regulated pilus (TCP), a type IV pilus, is an essential intestinal colonization factor. The *tcp* gene cluster which encodes TCP is clustered on a 39.5 Kb pathogenicity island (PAI) called the *Vibrio* Pathogenicity Island (VPI-1). A second PAI was recently described that is unique to *V. cholerae* O1 serogroup isolates, VPI-2 a 57.3 kb region that encodes genes involved in sialic metabolism (*nan-nag*), a neuraminidase (*nanH*), a type-1 restriction modification system and a Mu phage-like region. The 10 kb *nan-nag* region of the VPI-2 in *V. cholerae* encodes the genes required for the uptake and degradation of sialic acid, which is found in the human intestine as part of the mucin. The mucin scavenging properties of the genes encoded within the *nan-nag* region are thought to play a decisive role in the mechanism of action of the CT, since it may expose the GM1 receptors thus allowing the toxin to bind. Since VPI-2 is flanked by mobile elements, repeat sequences, and is intrinsically unstable, it is possible that VPI-2 is acquired by Horizontal Gene Transfer (HGT), which makes their evolutionary history especially intriguing. In this work, we are studying the underlying historical relationships between the different genes in the *nan-nag* region that is *nanA, nanE, nanK* among a wide range of bacteria. To carry this task out we have made use of several bioinformatics tools that enable us to find homologues of the *nan-nag* proteins in the databases, with the resulting alignments being used to reconstruct phylogenetic trees. Furthermore, BLAST analysis provided us with an insight into the variable presence of the *nan-nag* genes in completed *Vibrio* genomes such as *V. vulnificus* strains YJ016 and CMCP6, *V. parahaemolyticus* RIMD2210633, *V. fischeri* ES114 and *P. profundum* SS9. Phylogenetic trees for NanA, NanE, and NanK generated from assembled amino acid sequences from a range of bacterial species are compared with their respective 16S rRNA gene trees to determine possible HGT footprints. Phylogenetic trees based on NanE and NanK sequences are largely congruent with phylogenetic trees based on 16S rRNA among the species examined, with a number of interesting exceptions. The phylogenetic tree based on NanA showed a very different topology to the 16S rRNA gene tree for the same set of species. With these data the historical relationships of the genes belonging to the *nan-nag* region is determined and provide us with a glimpse of the erratic evolutionary history of this region among bacteria.

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G-9

Comparative genomics of two large plasmids present in the marine mollusc pathogen *Vibrio tapetis* type strain CECT4600

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The marine bacterium *Vibrio tapetis* is the causative agent of the brown ring disease affecting cultured Manila clams *Ruditapes philippinarum,* causing heavy economic losses in Europe but also in South East Asia. Previous investigations have demonstrated that *V. tapetis* strains constitute a homogenous group, sharing similar biochemical and antigenic properties. Further characterization of *V. tapetis* isolates using pulsed-field electrophoresis (PFGE) and plasmid profiling techniques showed that all the investigated strains harboured large plasmids. We identified two large plasmids in type strain CECT4600 of *V. tapetis*, our model organism, one predominant of about 90 kb in size (we called it pVT1) and a second one very low copy number which size was estimated to ~ 65 kb (pVT2). By analogy with other large plasmids found in other pathogenic or symbiotic *Vibrio* species, we speculated that these plasmids could be involved in the virulence properties of *V. tapetis*. To elucidate the potential functions encoded by these plasmids we have undertaken their complete genome sequencing. Preliminary analyses based on partial sequences revealed that pVT1 shared important homology with the conjugative plasmid pYJ016 (48.5 kb) from the human marine pathogen *Vibrio vulnificus*, while pVT2 is related to plasmid pJM1 (65 kb) from the fish pathogen *Vibrio anguillarum* encoding a siderophore system. An updated comparative genome analysis of these plasmids will be presented and discussed in terms of horizontal gene transfer and implications in evolutionary ecology.

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G-10

Detection of Vibrio cholerae in seafood using real time PCR

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Vibrio cholerae is the responsible of the severe dehydrating diarrhoeal disease cholera causing up to 120 000 deaths worldwide every year. One of the etiological agent's major protective antigens appears to be the O antigen and the enormous serological diversity with more than 200 described serogroups.
Today, we are in the seventh pandemy recorded in the history and only the strain O1 "classical" is implicated in six first pandemies and O139 "El Tor" in the seventh pandemy.
Nevertheless, non-O1 non-O139 strains were involved in foodborne diseases in developed countries. The disease is a gastroenteritidis and the reservoir was mainly crude seafood.
Vibrio cholerae is a mobile gram-negative bacterium and colonizes the human small intestine where it produces virulence factors that cause diseases. Two of the principal genetic elements were involved in pathogenesis: (i) the phage CTXΦ harbouring, among others, the *ctxA* and *ctxB* genes encoding the Cholera toxin and (ii) a pathogenicity island harbouring, among others, the *tcp* operon encoding the Toxin Coregulated Pili (TCP).
The aim of this work was to develop a real time PCR method in order to detect *V. cholerae* in seafood.
The first step was to choose a gene target. After Genbank examination, the *pomA* gene involved in the flagella biosynthesis was chosen *in silico*. Primers and probe were selected using the primer express™ software. The specificity and the sensitivity of the amplification were determined. Moreover, an internal PCR control was constructed. The real time PCR system was used to detect *V. cholerae* in artificially contaminated mussels. The detection limit was around 10 CFUs per gram. The positive samples were plated on TCBS on the characteristic colonies were checked for the present of the virulence genes: *ctxA* and *tcpA* both by classical and real time PCR.

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G-11

Comparison of Vibrio Genomes

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Currently there are six genomes from the *Vibrio* group that have been sequenced and publicly available, (five *Vibrio* and *Photobacterium profundum*). I will give a brief overview of the use of a variety of bioinformatic approaches to compare these 6 genome sequences to each other and to more than 250 additional bacterial genomes. The approaches include: 1.) Chromosome alignment; 2.) AT content in the genome and upstream of genes; 3.) Bias in oligomeric sequences towards the leading or lagging strand of the DNA; 4.) Repeats (local and global); 5.) organization of rRNA operons; 6.) tRNAs and codon usage; 7.) Third nucleotide position bias in codon usage; 8.) Amino acid usage; 9.) Promoter analysis; 10.) Annotation quality; 11.) Blast atlases; and 12.) Proteome comparision, including global regulators, sigma factors, and transcription factors, as well as secreted proteins and secretion systems.

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Disease & Epidemiology

D-1

The symbiosis polysaccharide (*syp*) gene cluster promotes symbiotic colonization and biofilm formation by *Vibrio fischeri*

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The light organ of the Hawaiian bobtail squid *Euprymna scolopes* is densely colonized by the marine bioluminescent bacterium *Vibrio fischeri*. Juvenile squid hatch from eggs uncolonized, but rapidly, within hours, become colonized by *V. fischeri* cells present in the environment. This symbiosis is specific: closely related *Vibrio* species, such as *V. parahaemolyticus*, fail to colonize the squid. At present, the factors contributing to this specificity are poorly understood. Symbiotic initiation requires motility, an outer membrane protein, and regulatory genes, including flagella and bioluminescence regulators. To increase our understanding of the initiation process, we searched a transposon mutant library for strains defective in initiating colonization. We identified 4 new genes required for symbiosis that each mapped to a cluster of genes on the small *V. fischeri* chromosome. Due to the predicted functions of these genes in polysaccharide biosynthesis, we have termed this region *syp*, for symbiosis polysaccharide. Mutation of the *syp* genes resulted in substantial defects (~1000-fold reduction) in the ability of *V. fischeri* to initiate the symbiosis. Investigation of *syp* gene function in the laboratory revealed no obvious defects in known symbiosis traits. We subsequently determined that transcription of the *syp* cluster did not occur under standard laboratory conditions, but could be induced by overexpression of a cluster-encoded response regulator gene, *sypG*. We anticipate that, like other response regulators, SypG is activated through a phosphorylation cascade initiated by a cognate sensor kinase in response to an environmental signal, such as might be present in the squid environment. In addition to domains predicted to be important for phosphorylation and DNA binding, SypG contains a σ^{54} activation domain. Such a domain provides the energy for open complex formation by RNA polymerase containing the alternative sigma factor, σ^{54}. Consistent with a role for SypG in activating σ^{54}-dependent *syp* transcription, we identified

potential σ^{54}-dependent promoter sequences and found that *sypG*-induced *syp* transcripts initiated 12-14 bases downstream of these predicted promoters. Furthermore, activation of *syp* transcription by SypG depended upon a functional copy of the gene encoding σ^{54}, *rpoN*. Due to the potential role of the *syp* genes in polysaccharide production, a characteristic important for biofilm formation by bacteria, we evaluated the ability of wild-type, mutant and *sypG*-overexpressing strains to form biofilms in culture. Although there was little difference in biofilm formation by wild-type and *syp* mutant strains, overexpression of *sypG* resulted in a dramatic increase in biofilm formation. This was true for cells grown in static culture, and even more so for cells grown with shaking, suggesting that aeration may play a role in biofilm formation by *V. fischeri*. The enhanced biofilm depended upon at least one *syp* member, *sypC*, which is predicted to encode an inner membrane protein. Finally, bioinformatic analyses revealed that the *syp* gene cluster is largely conserved in two related *Vibrio* species, *V. parahaemolyticus* and *V. vulnificus*, but not in a third, *V. cholerae*. Our future work will be directed at understanding whether the *syp* genes promote specific interactions of these organisms with their respective hosts, and if so, how these genes contribute to the observed specificity.

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D-2

Properties of vibrios associated with tropical Pacific corals

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Vibrios, including *Enterovibrio corallii*, *Photobacterium eurosenbergii*, *Vibrio coralliilyticus*, and *V.shiloi* have been associated with bleaching and necrotic disease in corals. Using culture, clone library and DGGE based molecular community analysis; we have recently shown that vibrios, including the above species, are also commonly associated with healthy corals. We investigated the possibility that temperature-regulated expression of virulence factors, such as extracellular enzymes, is a factor in the disease process. Bacteria were isolated from tissue slurries and mucus extracts of seven species of corals from the Great Barrier Reef and Hawaii using thiosulphate citrate bile sucrose (TCBS) and marine salts agar. *Enterovibrio corallii*, *Photobacterium eurosenbergii*, *V.coralliilyticus*, *V.fortis*, *V.harveyi*, *V.campbellii*, *V.rotiferanus*, *V.shiloi*, and *V.pelagius* were identified using 16S rDNA and rpoA sequencing, and with AFLP fingerprinting. We tested a large number of isolates for the production of extracellular enzymes, including cytolysins, proteases, and mucinases. Cultural conditions and temperature had a marked effect on the production and activity of these enzymes *in vitro*. The ecological role of these factors in the disease process is discussed.

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D-3

Association of vibrios with tissue necrosis in the pink sea fan *Eunicella verrucosa*

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There is currently considerable concern among marine conservation authorities regarding the health of colonies of the gorgonian octocoral *Eunicella verrucosa* (pink seafan) in coastal waters of southwest Britain. This species is on the 'red list' of threatened species and is one of the few marine organisms fully protected under UK legislation. In the last three summers, extensive outbreaks of necrotic disease have been observed by divers. In some cases there appears to be extensive destruction of tissue, resulting in exposure of the black *gorgonin skeleton and overgrowth by e*pibiotic invertebrates and algae. In summer 2004, we subjected samples of diseased and apparently healthy tissue to microbiological analysis by culture on a variety of media. No evidence of fungal infection was found, using two types of fungal isolation media. With the exception of one sample, all tissue clippings classified as diseased had higher concentrations of bacteria per gram of tissue (growing on marine salts agar, a general medium for isolation of marine bacteria). Use of thiosulphate-citrate-bile-sucrose agar (TCBS) showed that there diseased tissue contained a significantly greater mean concentration of presumptive members of the *Vibrionaceae* than healthy tissue. Forty eight bacterial colonies, initially identified as separate types on the basis of colony size and appearance were isolated. Eventually, 21 pure cultures of distinct types were obtained in pure culture and characterized using microscopic examination and biochemical tests (Microbact 12A/B and API20E systems). Using these methods, all but two of the cultures were confirmed as vibrios and preliminary identification results using partial 16S rDNA sequencing will be reported. Most isolates appear to be closely related to *Vibrio splendidus*. It is suspected that some isolates may form novel taxa. Many of the isolates were shown to be potent producers of proteolytic and cytolytic enzymes and it is suggested that these may be important factors in the induction of tissue necrosis. We will present evidence of the effects of temperature and other factors on the production and activity of these enzymes, and discuss their possible role as virulence factors.

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D-4

Development and production of preparations for cholera prophylaxis and diagnosis in the Russian Federation

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An oral preparation of cholera vaccine in tablets has been developed, licensed and manufactured at the Institute of "Microbe" in the Russian Federation. The vaccine consists of O antigen, (LPS), derived from Inaba and Ogawa serovariants as well as of detoxicated cholera toxin. The antigens are obtained from supernatant fluids of *Vibrio cholerae* strains M41 and 569B by means of ammonium sulfate precipitation followed by ultrafiltration on tubular fiber to purify the material. The lyophilized antigens are used to form the tablets of the preparation containing 10^5 BU (antitoxin binding units) or more choleragen-anatoxin and not less than 1:1000 O antigen as evaluated by the gel diffusive precipitation test with the specific serum. As shown by the active mouse protection assay, the immunogenic activity expressed through ED50 value is no less than 1:20000 part of a tablet. Three tablets form a single personal vaccination dose for an adult, 2 tablets are recommended for adolescents aged between 11 and 17 years, and one tablet is the dose used for children of 2 to 10 years of age. The vaccine induces the generation of vibriocidal and antitoxic antibodies, as well as coproantibodies found in the intestine. This high-level immunity lasts about 6-7 months. Clinical trials confirmed safe and non-reactogenic properties of the vaccine. Moreover, about 10000 people were immunized with this vaccine in Sakhalin Island in 2000 with not a single instance of adverse post-vaccinal response. On the basis of this vaccine, an efficient production technology was developed to manufacture vaccinal tablets against *V.cholerae* O139 serologic variant (RF Patent N 2159128) where serovariant Ogawa O antigen was substituted with the antigen derived from O139 strain cultures. The vaccine is now receiving a control trial. Further improvement of oral cholera vaccines is aimed at obtaining purest antigens and increasing their immunologic efficiency by addition of other protective cholera vibrio antigens (adhesion pili, enzymes, etc). A large group of preparations have been developed at "Microbe" Institute in Russia designed for cholera diagnosis, including several licensed sera for slide agglutination tests permitting O1 group vibrios Inaba, Ogawa, RO forms and serovariant O139 to be detected. Equine fluorescent immunoglobulins are strongly specific for O1 group *V. cholerae*, their working dilution being 1:8. The bacteriophage preparations manufactured are applied to detect classical and El Tor cholera vibrio biovariants, CDP phages - to assess El Tor vibrios virulence, ctx^+ and ctx^- phages can be used to detect epidemically dangerous El Tor strains, TEPV phages - for typing vibrios that are not agglutinated with vibrio O serum, and DDP phages to differentiate among the vibrios. Certified and widely used in Russian laboratory practices are specially devised PCR test systems to detect $ctxA^+$ gene determining *V. cholerae*

epidemic significance. Preparations for rapid detection and identification of cholera vibrios have been developed on the basis of dot-immunoanalysis which are now being actively introduced into practice. The manufactured commercially available preparations were tested in multiple trials to analyze materials from people and the environment during cholera outbreaks in Russia and adjoining countries, having given a good account of themselves as rapid and reliable means to reveal cholera vibrios.

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D-5

Recognition of various toxins from *Vibrio carchariae* and *Vibrio harveyi*

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Aquaculture is one of the most important industries for many countries and its farming style has shifted from extensive to intensive culture to bring more profit. Due to this intensification in aquaculture, problems in terms of pathogens have become very serious. Deteriorations in aquaculture particularly for prawn production are primarily caused by *Vibrio harveyi* (*V. harveyi*), one of the pathogens causing vibriosis in the main areas for world aquaculture production[1]. Thus, it is of significant importance in the aquaculture industry to control *Vibrio* and its toxin production.

Generally, *V. harveyi* is known as a bacterium which utilizes quorum sensing in cell-cell communication to express its luminescence. In *V. harveyi*, emission of luminescence and the expression of the lux operon are dependent on two auto regulators: N-(3-hydroxybutanoyl)-L-HSL(3-hydroxy-C4-HSL) (AI-1) [2] and a nonacyl-compoud (AI-2) [3], recently shown to be a furanone. During cell growth phase, luminescence initially lags well behind growth and subsequently increases at a significantly faster rate after the autoinducers have accumulated in the medium. It is a fact that some *V. harveyi* emit luminescence, while some do not. Until now the ability of *V. harveyi* to express luminescence is still unclear. *Vibrio carchariae* (*V. carchariae*) which is closely related to *V. harveyi* based on 16SrDNA sequence and share its luminescence characteristic was also used in this study. Among the *Vibrio* strains, *V. harveyi* isolated from shrimp farms in Thailand and Philippines did not emit luminescence. On the other hand *V. carchariae* isolated from shrimp farms in Thailand emitted luminescence and was observed to be very intense.

Regarding toxicity, it was reported that luminescence is clearly related to toxin production in *V. carchariae*[4]. However, in the case of *V. harveyi* isolated from shrimp aquaculture in

Thailand and Philippines, there were no luminescence but toxicity tests showed that their toxin production was higher compared to standard strains and to other *V. harveyi* (IFO15632, LMG10947, IFO11224). It was earlier reported that toxin expression in *V. harveyi* was mainly derived from phage, whose genes coding for toxin production was acquired by transduction. In addition, in the case of *V. cholera* and *P. aeruginosa*, toxin production was related to quorum sensing, suggesting that toxin expression is one of the quorum sensing phenomenon in some bacteria. Therefore, it might also be possible for *V. harveyi* to express toxin by quorum sensing.

In this study, extracellular proteins from *Vibrio* culture cell-free supernatant were detected using SDS-PAGE analysis. Results of SDS-PAGE analysis showed that there was a difference in molecular weight and amount of proteins between *V. harveyi* and *V. carchariae*. Both *Vibrios* showed one band approximately 38kDa (putative cysteine protease)[5], but amount of protein was much different. *V. carchariae* had higher amount of protein in 38kDa compared to that of *V. harveyi*. On the other hand, only *V. harveyi* isolated from shrimp aquaculture showed a band approximately 34 kDa putative haemolysin protein.

Moreover, to be clear with the toxin origin, the effect of protease inhibitors on pathogenicity was also investigated. It was found that light emission of pathogenic *V. carchariae* remarkably decreased after addition of protease inhibitors. According to previous reports, *Vibrio* haemolysin was thermo labile at 60 ![6]. In this study, toxin of non luminescence pathogenic *V. harveyi* was subjected to heat treatment to test if haemolysin was present. It was found that *V. harveyi* toxin was inactivated by heat treatment, unlike *V. carchariae* toxin which was not. To elaborate on this, results of haemolytic and cytotoxicity assay of *V. harveyi* isolated from shrimp farm showed higher toxicity compared to standard strains. On the other hand, *V. carchariae* isolated from shrimp farm showed low toxicity for the haemolytic assay but high toxicity for the cytotoxicity assay. Thus, it was suggested that protease toxin and luminescence in *V. carchariae* are related. In addition, based on the results of this study, it is concluded that *V. harveyi* produces various types of toxins.

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D-6

Impact of *Vibrio proteolyticus* on probiont uptake by *Artemia franciscana*

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Low survival rate of larvae, mostly due to diseases, is a well known phenomenon in larviculture. Until recently antibiotics did provide a temporary solution to counteract high mortalities. Due to a negative environmental impact, a fast increase of resistant pathogenic strains, as well as the presence of antibiotic residues in aquaculture products, alternative and more sustainable methods are looked for to counteract such pathogens.As in young larvae vaccination is not possible, research started up to use probiotics instead of antibiotics.
In this study probiont uptake experiments were done with *Artemia franciscana*. The pathogen *Vibrio proteolyticus* CW 8T2 and the probiont *Aeromonas hydrophila* LVS3-GFP were used to load the nauplii. The bacteria were applied separately, as well as in a mix, in order to investigate any competition. Concentrations in the *Artemia* medium reached on average 10^8 CFU/ml. Using fluorescent labeled bacteria the time needed for maximal uptake was investigated. It was then decided to collect the *Artemia* samples after two hours incubation. Ingested bacteria were quantified with Real-Time PCR as well as with the classical plate counting method. Results of both methods were compared. Several primer sets were tested for the quantification of *Aeromonas hydrophila*. A GFP primer set was giving the best results. For the quantification of *Vibrio proteolyticus* the primer sequences
F-132F: CACTCAGACCCACCAATTTTTCTTT and
R-192R: CCCCATCAAGGTGTCGATACCTC were successfully selected. As the number of bacteria detected with plate count method and real-time PCR were in most cases of the same order of magnitude, analyses were continued with the last method only. The competition between the pathogen *Vibrio proteolyticus* and the probiont *Aeromonas hydrophila* was quantified and will be discussed in detail.

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D-7

Molecular Typing: Randomly amplified polymorphic DNA (RAPD) studies to evaluate discriminatory abilities between virulent and avirulent isolates of *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus is the most common cause of seafood related gastroenteritis in South East Asia but not all strains of this species can be considered truly pathogenic. Three serotypes, O3:K6, O4:K68 and O1:K untypeable are responsible for a pandemic *V. parahaemolyticus* infection. Strains belonging to the O3:K6 clone have been responsible for large oyster associated outbreaks in the United States but have not been identified from either seafood or clinical cases in Europe. Most virulent isolates from clinical cases express *tdh* and *trh* genes, the genes responsible for thermostable direct haemolysin (TDH) and TDH-related haemolysin production. In addition, recently the presence of a type III secretion system has been implicated in the pathogenicity of *V. parahaemolyticus*. A significant proportion of isolates from seafood do not exhibit or express these genes and consequently the significance of strains isolated from the environment is not clear. This has necessitated the development of rapid identification and detection methods for virulent isolates. The use of randomly amplified polymorphic DNA (RAPD) has been applied extensively as a relatively quick and easy method to subtype bacterial isolates of the same species. In our study we have screened six 10-mer PCR primers, in pairs, to evaluate their ability to obtain discriminatory banding profiles in virulent and avirulent isolates of *V. parahaemolyticus*. From this we deduced a pair of primers to characterise 86 isolates of *V. parahaemolyticus* and closely related organisms of clinical and environmental origin from UK and Ireland. We will present extensive genomic fingerprinting data that shows a high degree of intra-species variation in banding patterns. The sensitivity of this RAPD method and its reproducibility will be demonstrated as well as an in depth cluster analysis using BioNumerics software.

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D-8

Phylogenetic diversity and ecology of *Vibrio aestuarianus*, a pathogen of oysters *Crassostrea gigas.*

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Pacific oyster Crassostrea gigas is regularly subjected to mortality outbreaks during summer. These summer mortalities have been observed for a long time in different countries, including Japan, USA and Brazil. In France, high mortality rates of Crassostrea gigas spat (30 to 50%) have been reported since 1991. In all these cases, mortalities occurred when temperature rose above 19°C and during the oyster reproduction period. Other numerous factors are also suspected to start or favor these mortalities. In order to determine the implication of potential pathogenic bacteria in these outbreaks, an epidemiological study was performed in different areas along the French coasts during 2001-2005 summers. Several strains were isolated from hemolymph of moribund animals. Preliminary genotypic characterizations showed that 65% of these isolates were related to the *Vibrio aestuarianus* species, 30% were related to the *Vibrio splendidus* polyphyletic group and 5% belonged to other species. Previous works demonstrated the association of *V. aestuarianus* with marine mollusks, but any pathogenicity study has never been performed until now.

Fourteen strains of *Vibrio aestuarianus* were therefore selected for pathogenicity and phylogenetic studies. The strain ATCC35048t, isolated in Oregon, was used as reference strain. Experimental infections showed a great variablity in the virulence of the different isolates. Three strains were not pathogen, while three isolates were able to induce 20-50% mortalities in oysters and five strains induce above 50%.

Different genes including 16S RNA, GyrB and ToxR were cloned and sequenced for a Multi Locus Sequence Typing. All isolated strains presented above 99% similitude on these three genes. This was confirmed by quantitative DNA-DNA hybridizations, with hybridization rates above 80% for isolates originating from oysters. By contrast, the reference strain ATCC35048t displayed only 50 to 77% hybridization with these isolates, demonstrating therefore a highly conserved genotype between these 14 isolates of *V. aestuarianus*.

We are currently studying the ecology of *V. aestuarianus* using colony-blotting and PCR procedures. A preliminary study performed in microcosm using a virulent isolate demonstrated that the bacteria were not able to maintain in seawater as cultivable form. Moreover, they could not be detected in hemolymph of healthy oysters but it multiplied in diseased oysters suggesting a potential strict pathogenic character. PCR and Colony Blotting let us to detect the specie in mud located under oysters on different places of french coast..This let us think that sediment could be the reserve of *Vibrio aestuarianus,* next relayed by infected and dying oysters.

To conclude, these results demonstrated the pathogenicity of *V.aestuarianus* to C. gigas oysters and the highly genotypic conservation of the specie. Prelimilary study provide new insights into the ecology of this species.

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D-9

Real time PCR for sensitive detection of total and pathogenic *Vibrio parahaemolyticus* in estuary water in Southern Brittany, France.

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Vibrio parahaemolyticus is a bacterium that is indigenous to coastal waters. Although few environmental strains of *V. parahaemolyticus* are capable of causing human disease and although the overall disease incidence is relatively low compared to other foodborne pathogens, an increasing number of *V. parahaemolyticus*-associated outbreaks has been documented. The most recent outbreak reported 3000 cases of gastro-enteritis and one death in Chili (Feb. 2005) and was mainly attributed to El niño and the subsequent increase of sea surface temperature. In 2001, 100 cases were reported in France; this outbreak was attributed to the presence of pathogenic *V. parahaemolyticus* (*tdh*) in mussels originating from Ireland and processed in a shellfish plant in Brittany .

Real time PCR (SYBR Green) was developed and used for monitoring *V. parahaemolyticus* in a shellfish growing area from Southern Brittany. The aim of the study was to survey the appearance of total and potentially pathogenic *V. parahaemolyticus* in water and in shellfish cultivated in this area in relation with water temperature and salinity – and ultimately, to provide shellfish growers with these information and advises for stock management ect. Probes recording the environmental parameters every 30 minutes were set at two sites. Water and mussel samples have been collected from the same two sites at 2 week intervals since the beginning of May 2005 and were analysed for the presence of *V. parahaemolyticus*. Classical and molecular methods were used simultaneously for the detection of *V. parahaemolyticus*. Two hundred ml seawater samples were filtered and analysed directly, or after an enrichment step, by culture on selective media (TCBS, CHROMAgar Vibrio), PCR and real time PCR. Housekeeping gene *toxR* and virulence gene *tdh2* were selected to detect total and some of *V. parahaemolyticus* pathogenic strains, respectively. New sets of primers are being designed for the detection of the other virulence gene, *trh*.
Amplification of the *toxR* gene - indicating the presence of total *V. parahaemolyticus* - was detected in PCR in only one of the 8 water samples whereas it was found in 7 of the 8 samples

by real time PCR. Between 69 and 2150 CFU equivalent per 100ml of estuary water were detected in one site and between 0 and 5190 in the other site. The analysis of enrichment broth from the above water samples by real time PCR confirmed the absence of *V. parahaemolyticus* in the water collected at the beginning of May 2005 at one of the sites. The water temperature was below 12°C at the time of the sampling. *Vibrio parahaemolyticus* was detected in mussels from only one site. The *tdh2* gene was not amplified by real time PCR in any of the enrichment suspensions thus, indicating the absence of potentially pathogenic (*tdh2*$^+$) strains of *V. parahaemolyticus* in the tested samples.

These data indicated the detection of total *V. parahaemolyticus* in real time PCR when no bacteria was detectable by culture or direct PCR. This sensitive technique will be used during the summer and fall 2005 to further survey the variation in total *V. parahaemolyticus* abundance and control the possible appearance of potentially pathogenic strains in this shellfish growing area.

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D-10

Searching for genetic markers of virulence in *Vibrio nigripulchritudo*, a *Litopenaeus stylirostris* shrimp pathogen isolated in New-Caledonia.

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Since 1997, a new pathology seasonally occurs in new caledonian shrimp farms during the warm season. Diseased *Litopenaeus stylirostris* shrimp suffer from a systemic vibriosis which was attributed *to Vibrio nigripulchritudo*. At the present time, only two farms among 17 are affected by the so called "summer syndrome". Spreading of the disease to other shrimp farms would undoubtedly threaten the sustainable development of new caledonian shrimp industry. In such a context it appears urgent to develop reliable diagnostic tools to detect virulent *V. nigripulchritudo* strains. Indeed, if *V. nigripulchritudo* strains are easily identifiable taxonomically using phenotypic characters, experimental infections revealed that only some of them are virulent. Furthermore these strains were found undistinguishable from avirulent ones using classical biochemical tests.
The aim of this work was to genetically characterize virulent *V. nigripulchritudo* strains, analyzing the genetic diversity of 24 virulent or avirulent strains belonging to this vibrio species and to identify genes present only in one virulent strain. Firstly, the results of molecular typing obtained in Multilocus Sequencing Typing (MLST) and in PCR Restriction Fragment Lengh Polymorphism (PCR-RFLP), highlight the existence of a group characterized by a small degree

of genetic variability and containing only strains displaying moderate to high virulence. Secondly, two phylogenetically closed strains, one highly virulent SFn1 and the other avirulent SFn118 were selected and treated by Substractive Suppression Hybridization (SSH) in order to characterize specific genes of the virulent strain. A total of 470 genes fragments found only in SFn1 genome were obtained and further identified using BlastX software. After validation, these genetic markers would be of great interest to develop in next future relevant diagnostic tools as macroarrays for epidemiological studies.

Keywords : *Vibrio nigripulchritudo*, *Litopenaeus stylirostris*, MLST, PCR-RFLP, SSH.

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D-11

Detection and characterisation of total and haemolysin-producing Vibrio parahaemolyticus *in bivalve molluscan shellfish from U.K growing waters*

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Vibrio parahaemolyticus food poisoning, associated with seafood consumption has been identified in many countries. Outbreaks are most commonly reported from countries with both a high ambient water temperature and where seafood is consumed raw. Thus infections are predominately limited to sub-tropical regions of the United States and Southeast Asia, where *V. parahaemolyticus* is considered to be the primary cause of seafood related gastroenteritis. In Europe, infection is mainly travel related, although some outbreaks have been due to the recontamination of cooked seafood by raw infected food or contaminated seawater. *V. parahaemolyticus* has, however, been reported from the marine environment and shellfish in several European countries. In 2004, an outbreak of O3:K6, *V. parahaemolyticus*-associated gastroenteritis was reported in Spain. Seventy-six cases were recorded following the consumption of edible crabs (*Cancer pagurus*) by wedding guests. The crabs had been harvested in the U.K.

Currently, scant information exists across the EU on the prevalence and epidemiological significance of *Vibrio* species in seafood entering or harvested in European states. Generally methods for the detection and characterisation vary widely within Europe and it is difficult to assess the public health significance of the isolations. This is particularly the case for *V. parahaemolyticus* where it is estimated that only up to 5% of environmental isolates are capable of expressing the thermostable direct (TDH) and related haemolysins (TRH) that are associated with gastro-enteritis. Thus testing regimes that report total *V. parahaemolyticus*

or even total vibrios may lead to unnecessary consumer alarm and do not constitute the most effective means of public health protection.

This study describes the detection and characterisation of total and haemolysin-producing *V. parahaemolyticus* in bivalve molluscan shellfish and crustacea from U.K and Irish growing waters. Seventy one (ad hoc) samples were collected from commercial fisheries and examined for the presence of *V. parahaemolyticus* using conventional microbiological and molecular methods (*toxR*-targeted PCR). The occurrence of potentially pathogenic strains, as determined by detection of *tdh* and *trh* genes encoding for TDH and TRH respectively, was confirmed using PCR. A standardised protocol for Pulse Field Gel Electrophoresis (PFGE) was developed and used to characterise *V. parahaemolyticus* isolates. The clinical significance of strains isolated from seafood's was assessed by comparisons with strains of known epidemiological importance.

Between 23 and 31% of samples were positive for *V. parahaemolyticus* dependent upon the method used. Classical microbiological methods consistently produced higher incidence of detection than molecular methods. Nine strains were found to be *tdh* positive. PFGE patterns showed that strains isolated in U.K and Irish seafood were closely related to each other but showed little relationships to pandemic clones O3:K6 and O3:KUT. Further studies are underway to establish the homology between UK and Irish isolates with clinically significant European clones.

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D-12

Mass mortality of Japanese abalone *Haliotis discus hannai* caused by *Vibrio harveyi* infection

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Part of *Vibrio harveyi* strains (including junior synonym of *V. carchariae* and *V. trachuri*) are pathogens in a wide variety of marine fish (turbot, shark, grouper and so on) and shellfish (shrimp, abalones, and so on). We also identified *V. harveyi* as a causative pathogen of a mass mortality of Japanese abalone *Haliotis discus hannai*. Abalone aquaculture has been established 1950's in Japan to sustain rapidly deceasing resources of the wild abalones. A Japanese abalone, *Haliotis discus hannai*, is traded in the most expensive value (ca 100 US$ per kg) as Ezo abalone. Abalone farms for *H. discus hannai* built on beginning of 1980's in Hokkaido (north part of Japan) are capable to produce 5 million of abalone juveniles.

The mass mortality of the cultured abalone, however, has been occurred since late 1980's in Hokkaido area, especially on summer. Signs of the abalone disease are unusual migration to water surface and a swelling lesion in internal organ due to an accumulation of body fluid. A total of 5-10% loss has been recorded in every summer. We isolated vibrio strains showing swarming motility from the body fluid of the dead abalones. The swarming vibrios composed of 40% of the microflora. The representative strains of the swarming vibrios were phenotypically and phylogenetically characterized, and identified as *V. harveyi*. LD50 of the *V. harveyi* strain was estimated at $6.4X10^5$ CFU/g abalone by the injection trial at 20°C. No infection was observed in the injection trial of the *V. harveyi* strain at 15°C. Using a currently developed species-specific primer set for detecting *V. harveyi*, abundance of *V. harveyi* in costal seawater (10^3-10^4 CFU/L), supply water (10^2 CFU/L), and gill of abalones (10^2 CFU/g gill) were detected before the outbreak in 2004.

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D-13

Characterization of virulence factors in *V. cholerae* non-O1, non-O139 environmental and clinical isolates

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Vibrio cholerae O1 and O139 causes diarrhoeal disease in humans by secreting cholera toxin (CT). Molecular analysis has revealed that in addition to genes encoding for CT, all strains capable of causing cholera invariably carry genes for a colonization factor known as toxin-coregulated pilus (TCP). The non-O1, non-O139 strains, which are predominantly isolated from aquatic environment, are largely non pathogenic in nature, although some of these are known to cause sporadic cases or occasional outbreaks of diarrhea in humans. *Ctx* and *tcp* genes have been shown to be present in pathogenic strains of non-O1 non-O139 *V. cholerae* of both clinical and environmental origins. However, strains of *V. cholerae* that do not produce CT have been isolated from patients with both intestinal and extraintestinal infections. These strains showed fluid accumulation in mice, proteolytic, hemolytic and cytotoxic factors which have been reported as potential indicators of pathogenesis. Moreover, in several isolates of *V.cholerae* hemolysins as TDH, TRH and TLH have been discovered. The present study was undertaken to explore the virulence factors of clinical and environmental strains of non O1, non O139 *V. cholerae*. A total of 25 strains, 2 of clinical and 23 of environmental origin, were included in the study. Environmental strains were isolated from samples of seawater and mussels, collected in the Adriatic sea, in front of the Ancona Province's coast (Marche Region, Italy). Clinical strains were isolated from patients with diarrhoeal disease. The isolates were identified by a biochemical standardized protocol and the sierotyping was performed by

O1 polyvalent and O139 antisera *V. cholerae*. To assess their virulence, all isolates were tested for cytotoxicity on Vero cells (CCL 81), protease, lipase, elastase, hemolytic activities and for the presence of *ctx, tcp, tl, tdh, trh* genes. The intestinal and extraintestinal colonization, fluid accumulation and lethality of the isolates were tested by the suckling mouse model of infection. As control ATCC 9459 *V.cholerae* strain was used. A lot of environmental isolates showed protease (62%), lipase (87%), elastase (62%), cytotoxic (25%) activities. The clinical isolates showed lipase and cytotoxic activities but not protease and elastase. The cytotoxic effect, characterized by the rounded and detached cells, was evident 3 h after treatment. All environmental and clinical strains were hemolytic in sheep blood agar. All isolated, when orally inoculated in infant mice were able to colonize the intestine but not to induce fluid accumulation; only cytotoxic strains were lethal and able to induce extraintestinal colonization. *Ctx, tcp, tl, tdh* and *trh* genes were never discovered in the isolates. The control strain showed cytotoxic, hemolytic, protease, lipase, elastase activities in vitro, and intestinal and extraintestinal colonization, fluid accumulation and lethality in vivo. This investigation confirm that *V. cholerae* is able to produce virulence factors other than CTX, TCP, TDH, TRH and TLH. Moreover, our results in the animal model suggest the virulence to be multifactorial in that: a) intestinal colonization was evident in all strains b) extraintestinal colonization and lethality were only evident in the cytotoxic strains c) the fluid accumulation, caused by CTX and TCP, was never underlined. Further investigations are required to explain the role of the different virulence factors in the pathogenesis of the human infections by non O1, non O139 *V. cholerae*.

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D-14

Functional analyses of the toxin-coregulated pilus protein TcpA from toxigenic *V. cholerae* isolates.

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Cholera, caused by toxigenic *Vibrio cholerae*, is a severe and sometimes lethal human diarrheal disease that can occur as spreading epidemics. *V. cholerae* belonging to the O1/O139 serogroups are generally considered to be the only causative agent of epidemic cholera. *V. cholerae* non-O1 and non-O139 serogroups are associated mainly with sporadic cases of diarrhoea and extra intestinal infections. Toxigenic strains of *V. cholerae* are found to possess two essential genetic factors, toxin-coregulated pilus (TCP) and cholera enterotoxin (CT), both of which are critical determinants of the pathogenicity of *V. cholerae*. TCP is a polymer of repeating subunits of the major pilin protein TcpA and functions both as an essential colonisation factor and as a receptor for CTXϕ, the filamentous phage that encodes CT. We are currently investigating the significance of genetic variation at the *tcpA* locus and potential role(s) this may play in the pathogenesis of the disease. We are using several molecular approaches including both isogenic allele exchange and Yeast Two Hybrid interaction assays, with the mode of action of TCP in CTXϕ receptor binding and intestinal colonisation providing the major focus of this work.

The main symptoms of cholera, profuse secretory diarrhea, are caused by the interaction between cholera toxin, an A-B type toxin that is encoded on a filamentous bacteriophage CTXphi and its host receptor, the type IV pilus TCP. It has been inferred from some studies that the pIIICTX protein of CTXphi interacts with the TcpA major pilin protein of TCP. To identify the proposed pIIICTX (CTXphi) and TcpA (host cell) protein interaction and assess its specificity, we will use the CytoTrap® Yeast Two-Hybrid System from Stratagene. The CytoTrap system is based on reconstitution of the Ras signaling pathway and uses the novel yeast strain cdc25H, which contains a mutation in the guanyl nucleotide exchange factor cdc25 gene (the yeast homologue for hSos). The hSos gene product can complement the cdc25 gene, permitting growth at 37ºC, as long as hSos is localized to the membrane via a protein-protein interaction. The target protein is expressed as a fusion with the Src myristylation signal, which anchors it to the cytoplasmic surface of the cell membrane. The bait protein is fused with hSos. When bait and target are co-transformed into cdc25H yeast, only colonies containing a specific protein-protein interaction grow at 37ºC. We are interested in exploring the versatility of the yeast two-hybrid system in identifying bacteriophage-bacterial interactions by using a pIIICTX protein library as target and diverse TcpA proteins as bait to determine quantitative and qualitative interactions of these proteins.

Furthermore, the identification of several distinct alleles of the *tcpA* gene within different *V. cholerae* isolates has led us to explore the effect these have on colonisation and CTX-binding through the construction of several mutants in an isogenic background. Alleles from five strains will be introduced into *V. cholerae* O395 strain using the suicide plasmid pDS132, thus replacing the wild type *tcpA* allele and generating double crossover mutants. These mutants will subsequently be used in further studies including colonisation and CTXϕ transduction.

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D-15

Cholera outbreak in KwaZulu-Natal, South Africa: 2000 - 2004

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From August 2000 to the end of 2004 there have been 158 000 cholera cases reported in the mostly rural and economically impoverished province of KwaZulu-Natal, South Africa. The case fatality rate (CFR) was 0.36%, which to date is the lowest compared to previous South African epidemics. This has been mainly due to the prompt and effective inventions, which the KwaZulu-Natal Department of Health initiated. The Department's GIS Unit developed a spatially enabled database, which was used to capture patient information. Due to the fast turn around time and the wide range of maps and reports that could be produced, the database formed an integral part of the intervention strategy and helped with prioritising and directing limited resources. The spread of the disease seamed to be related to factors such as temperature and rainfall, water supply and sanitation services, availability of medical care and the movement of people during the Christmas holiday period. The spread may therefore be due to a complex interaction of environmental, social and / or infrastructural problems. By using the spatial and temporal information captured in the database, in-depth studies to investigate the factors responsible for the rapid and persistent spread of the outbreak could be initiated. This is the first time that information of this detail is available in South Africa for the investigation of a large epidemic.

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D-16

Detection and characterization of *Vibrio parahaemolyticus* in seafood using real time PCR.

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The genus Vibrio includes Gram-negative bacilli, anaerobic facultative, oxidase positive and mobile. These bacteria are halophylic meaning that NaCl stimulates their growth. Among this genus, three species were responsible for food poisoning due to the ingestion of seafood. *Vibrio cholerae* the cholera agent, *Vibrio vulnificus* responsible for septicaemia and *Vibrio parahaemolyticus* responsible for gastroenteritidis. The clinical signs of *V. parahaemolyticus* intoxication are nausea, fever, vomiting, and headaches… The major virulence factors include the thermostable direct haemolysin (TDH) and the TDH-related haemolysin (TRH). Moreover, all *V. parahaemolyticus* isolates produce a thermolabile haemolysin (TL).
The *tl* gene was used as a target for specific real time PCR in order to detect *V. parahaemolyticus* is seafood. The specificity and the efficiency of the PCR were checked. Moreover, an internal PCR control was constructed. The PCR was applied on artificially contaminated mussels and shrimps. The detection limit was between 1 and 10 CFUS/25g.
Moreover, the *V. parahaemolyticus* colonies were typed using a SybrGreen multiplex real time PCR for *tdh* and *trh* genes. The discrimination was based on the difference on melting temperature.
Therefore, a two-steps strategy was proposed: a first screening using the *tl* gene probe followed by a specific typing of characteristic colonies using the *tdh-trh* multiplex PCR.

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D-17

Molecular analysis and significance of El Tor strains of *Vibrio cholerae* O1 that carry some genes of the classical biotype

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Two biotypes of *Vibrio cholerae* O1 namely classical and El Tor are recognized, which can be distinguished from each other based on conventional phenotypic, and more recently, described genotypic traits. These include hemolytic properties, sensitivity to polymixin B and biotype specific phages, Voges Proskauer test, distinct ribotype patterns and the type of repressor gene (*rstR*) of the cholera toxin prophage. There is firm evidence that the fifth and sixth pandemics of cholera were caused by the classical biotype while the seventh is caused by the El Tor biotype, which has now completely replaced the classical biotype. The biotype status of strains of *V. cholerae* O1 that caused earlier pandemics are not known but assumed to be classical. New variants of *V. cholerae* O1 associated with sporadic cholera in Matlab, Bangladesh were shown to possess combination of traits of classical and El Tor biotypes by us in 2002 (Nair *et al.*, 2002). The conventional phenotypic and genotypic traits including the type of *rstR* gene in the CTX prophage failed to categorize these strains into classical or El Tor biotypes. In an effort to understand the distribution of the *rstR* gene of the classical and El Tor types, we analyzed a larger number of *V. cholerae* O1 and O139 strains isolated over the past eight years in Matlab. PCR surveillance of the *rstR* gene of the CTX prophage in *V. cholerae* O1 and O139 showed the low presence of the *rstR* of the classical type in El Tor strains of *V. cholerae* (Nusrin *et al.*, 2004). We then used pulsed field gel electrophoresis to analyze the restriction patterns of the whole genome of these hybrid strains (Matlab types I, II, III), to understand the level of genomic similarity between the hybrid Matlab strains and the classical and El Tor biotypes. Dendogram constructed by unweighted pair-group method using arithmetic averages generated from *Not*I restriction patterns of whole chromosomal DNA grouped these strains into two major clusters that were found to be similar but not identical to either of the biotypes. Strains that clustered with the classical biotype appear to have been derived from the classical strains (Safa *et al.*, 2005). The Matlab variants of toxigenic *V. cholerae* O1 strains assumed greater significance when detailed phenotypic and genotypic analysis of 40 strains of *V. cholerae* O1 isolated from Beira, Mozambique, revealed that the strains displayed typical traits of the El Tor biotype but carried the classical CTX prophage (Ansaruzzaman *et al.*, 2004). Comparative genomic analysis using microarrays based on the whole genome sequenced O1 El Tor strain N16961 have identified genes unique to the classical strains, pre-seventh pandemic El Tor strains and genes specific to seventh pandemic El Tor

strains (Dziejman et al., 2002). Detailed molecular analysis of the Matlab and Mozambique variants of *V. cholerae* using a genetic screen showed that the hybrid variant strains are basically El Tor types that have acquired some genes of the classical biotype. At this time, it is not certain whether such hybrid genomes will improve the pathogenic fitness of the strain or will have an impact on genomic stability or enhance their epidemic potential.

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D-18

Comparative phenotypic and genetic characteristics and virulence properties of *Vibrio tapetis* and related strains isolated from molluscs and fishes suffering vibriosis

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The marine bacterium *Vibrio tapetis* is the causative agent of the brown ring disease affecting cultured and wild populations of Manila clams, *Ruditapes philippinarum* and *Ruditapes decussatus* in Europe, North of Africa and in Asia. Along the European Atlantic coast, from Norway to Marocco, 30 *Vibrio tapetis* strains or related to this species have been isolated since 1988. They were originated from diseased clams or other molluscs. More recently, *V. tapetis* have been also implicated in fish vibriosis. A comparative study was undertaken to assess and compare their phenotypic, genetic and *in vivo* virulence characteristics. All *V. tapetis* isolates are grouped in a unique and homogeneous phylogenetic clade. Moreover, all *V. tapetis* strains were pathogen for the Manila clam *Ruditapes philippinarum* although their virulence varied according to strains.
V. tapetis pathogenicity mechanisms and their modulation were also studied. A cellular approach allowed us to show strong cytotoxic activities of this bacterium towards clam hemocytes. Indeed, *V. tapetis* can cause morphological alterations of hemocytes (cell rounding and swelling), induce inhibition of their adhesion capacities and of their oxidative metabolism and ultimately lead to hemocytes death. Molecular approaches were performed to identify virulence gene in *V. tapetis* strains. By using degenerated primers design for hemolysin genes of Vibrios, a lecithinase gene was identified in all *V. tapetis* strains. It is the first putative virulence gene identified in *V. tapetis*. To identify other genes in relation to the infection process, a subtractive bank, between two *V. tapetis* strains presenting high degree of differences in pathogenicity, is in progress. Another interesting research track is the omnipresence of large plasmids in all the virulent strains of *V. tapetis* investigated so far. By analogy with other known vibrioses (in human and fish), we assumed these plasmids could be involved in the virulence properties of this bacterium. We therefore started the complete sequencing of two large plasmids (~90 kb and ~ 65 kb respectively) present in the type strain CECT4600. An updated comparative genome analysis of these plasmids will be presented (as poster) during this conference, in addition to the present communication.
All the work accomplished on *V. tapetis* strains characterization brought essential knowledge on biogeography and phylogeny of this pathogen. More, by combining experimental infection, cellular and molecular approaches the present study contributed to a better understanding of *V. tapetis* pathogenicity and of interactions mechanisms with clam immune cells.

D-19

Non-cholera Vibrios infections in France, 1995-2004: ten years of surveillance

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Since 1995, the French National Reference Center for *Vibrios* and Cholera (CNRVC) at the Institut Pasteur, Paris, has maintened a voluntary surveillance system for non-cholera *Vibrio*

infections in France. Clinical data, including information about underlying illnesses, and epidemiological data on seafood consumption and exposure to seawater before illness, are systematically collected for each culture-confirmed non-cholera *Vibrio* infection reported to the CNRVC. This report summarizes the human non-cholera *Vibrio* isolates reported to the CNRVC the last ten years, from 1995 to 2004. A total of 93 cases of culture-confirmed *Vibrio* illnesses were reported. These *Vibrio* infections could be categorized into several syndromes: gastroenteritis with or without secondary septicemia; wound infections with septicemia; primary septicemia with no evidence of previous gastroenteritis or wound infection; and miscellaneous suppurations. *V. cholerae* (non-O1/non-O139) was the most frequently reported *Vibrio* species isolated (47.3 % of patients) and was associated with gastroenteritis with or without septicemia, wound infections, and septicemia. *V. parahaemolyticus*, which was mostly isolated from gastroenteritis, and *V. alginolyticus,* which was mostly isolated from miscellaneous suppurations, were each isolated from 18.3 % of patients. Among the 17 *V. parahaemolyticus* isolates, five belonged to the new pathogenic O3:K6 pandemic clone. *V. vulnificus* was isolated from 11.8 % of patients, mostly from severe wound infections and septicemia. Among the 93 patients, 65 (70%) were hospitalized and 8 (8.7 %) died. Seven of the eight patients who died presented underlying illnesses. Patients with underlying medical conditions were more likely to develop septicemia. Among patients for whom information was available, 80.5 % were contaminated in France with the others acquiring the infection abroad. Most infections were associated with eating seafood in the seven days before illness, contact with seawater or handling seafood. *Vibrio* illnesses were correlated with the warmer months of the year — from May to October. It is difficult to monitor *Vibrio* illnesses because these infections are not notifiable and only cases associated with severe syndromes are reported. Indeed, the official surveillance authority estimated that the number of cases reported by the National Reference Center was representative of severe *Vibrio* infections only. However, despite the low number of *Vibrio* infections reported in France, these pathogens should be included in microbiological surveillance systems because of the increase in the number of people susceptible to infection, the severity of infections and the recent introduction and probable implantation of a pandemic clone of *V. parahaemolyticus* in France.

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D-20

Detection and quantification of *V. cholerae* and *V. parahaemolyticus* in pure cultures, oysters and water samples by real-time PCR

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We have developed a real-time PCR method to detect and quantify directly in artificially contaminated environmental samples two medically important *Vibrio* species, *V. cholerae* non-O1/non-O139 and *V. parahaemolyticus*. The assays used oligonucleotide primers specific to the species and targeting sequences of the 16S-23S rRNA intergenic spacer regions for *V. cholerae* and the R72H sequence for *V. parahaemolyticus,* with detection by the SYBR green method. We optimized the MgCl2 concentration and developed DNA external control for validation of the test results. The best results were obtained with samples treated with Instagene matrix (Bio-Rad). The sensitivity was about 50 CFU in the reaction mixture when tested on fresh water seeded with *V. cholerae* or on oyster mantle fluid seeded with *V. parahaemolyticus*. The real time SYBR Green PCR assay was also optimized to detect the presence of the cholera toxin genes of *V. cholerae*, and the thermostable direct hemolysin (*tdh*) and the thermostable related hemolysin (*trh*) genes, markers of *V. parahaemolyticus* pathogenicity. In contrast to results obtained with cholera toxin, we could not confirm the specificity of various primers described in literature for *tdh* gene detection. Indeed, was also observed *tdh*-amplification with strains of *V. cholerae* and *V. hollisae*. The real-time PCR assay described here for evaluating the population of *V. cholerae* and *V. parahaemolyticus* in seeded environmental samples provides good sensitivity and is less time consuming than conventional methods (MPN) or other PCR protocols. Further experiments will improve the limit of detection for the quantification of *V. cholerae* and *V. parahaemolyticus*. We will also study *tdh* specific primers to be used to detect directly pathogenic *V. parahaemolyticus* in environmental samples.

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D-21

Vibrio carchariae detection and interaction with *Haliotis tuberculata* haemocytes.

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Vibrio carchariae has been isolated in the abalone *Haliotis tuberculata* during the mass mortality episode in 1998. This Vibrio is a subspecies of *Vibrio harveyi*. For the first time, this study describes and compares *Haliotis tuberculata* immunological parameters with other better known species such as clams, in absence and presence of several Vibrios.

The goal of this new research topic is to put in light the direct implication of this bacterium in ormer disease and to elucidate the exact mechanisms of interaction between Vibrios and *Haliotis tuberculata*.

Firstly, the project aimed at detecting the pathogen in the abalone environment thanks to specific oligonucleotides primers directed on the *Vibrio harveyi* toxR sequence.

Secondly, the *in vitro* immune response against Vibrios was analysed. The immune parameters were evaluated in presence of *Vibrio carchariae*, and compared with other Vibrio strains naturally present in sea water or known to be infectious for other molluscs. Immune response was estimated by the measure of haemocytes viability percentage, adhesion capacity (CNA), phagocytosis activity and production of reactive oxygen species (ROS) by flow cytometry.

This work is the first step in the research project aiming at studying *Vibrio carchariae* infection mechanisms and *Haliotis tuberculata* immunological response *in vivo*.

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D-22

Crystal structure of a monoclonal antibody directed against an antigenic determinant common to Ogawa and Inaba serotypes of *Vibrio cholerae* O1

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Cholera remains a major public and individual health problem. According to the WHO, the number of people susceptible to cholera due to socioeconomic problems has dramatically increased worldwide, creating favorable conditions for a global cholera crisis. Although improvement in hygiene is an efficient

method for the long-term control of cholera, in many developing countries this is still a distant goal. Hence, there is an urgent need for cholera vaccines that can confer reliable and long-term protection against *Vibrio cholerae* O1 and *V. cholerae* O139 in all age groups, including children less than five years of age. We have shown in an experimental model of cholera in neonatal mice, that immunoglobulin G (IgG) monoclonal antibodies directed against the lipopolysaccharide (LPS) of *V. cholerae* O1, are immunoprotective. We then began developing conjugate vaccines comprising the polysaccharide moiety of the LPS (pmLPS) of *V. cholerae* O1 and *V. cholerae* O139 covalently linked to a carrier protein, which should induce a long-lasting, thymus-dependent immune response. We have already prepared a conjugate composed of the polysaccharide of *V. cholerae* O139 and have shown that this vaccine does indeed induce a protective thymus-dependent response in mice. According to literature, *V. cholerae* O1 LPS contains at least two antigenic determinants. One determinant is present only on Ogawa cells. The crystal structure of the Fab fragment of a monoclonal antibody directed against this antigenic determinant has been determined both in its unliganded form and in complex with synthetic fragments of the Ogawa O-specific polysaccharide. The second known determinant is common to Ogawa and Inaba serotypes and it has been suggested that both the core and/or the O-specific polysaccharide of the LPS are involved in this antigenic determinant. To induce antibodies directed against both Ogawa and Inaba serotypes of *V. cholerae* O1, we prepared a series of conjugates of the polysaccharide moiety of the lipopolysaccharide (pmLPS) of *V. cholerae* O1, serotype Inaba, linked to Tetanus Toxoid. Although these conjugates were antigenic, none elicited a protective thymus-dependent response in mice. This result may be due to the alteration of the antigenic determinant common to the Ogawa and Inaba serotypes of *V. cholerae* O1 during the preparation of the conjugates. Our attempts to develop a rapid diagnostic test for cholera led to the development of a monoclonal IgG1 antibody specific for both Ogawa and Inaba serotypes. To understand better the fine structure of the antigenic determinant, we crystallized the Fab fragment of this monoclonal antibody and determined its crystal structure at 2.5 Å resolution using molecular replacement methods. The crystal structure revealed the presence of a putative monosaccharide-binding cavity in the recombination site, suggesting that the antibody may specifically bind a terminal sugar from the LPS. Molecular mechanics calculations and co-crystallization trials of different Fab-carbohydrate complexes are currently in progress to identify the actual determinant.

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D-23

New strategies for the isolation and identification of the eel and human pathogen *Vibrio vulnificus* serovar E

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Vibrio vulnificus serovar E (VSE) is a pathogenic bacterium distributed worldwide, which produces both epizootics/outbreaks of high mortality in cultured eels and sporadic infections in humans. This serovar belongs to the biotype 2 of the species, which currently includes the strains which are virulent for eels. The hazard that virulent VSE (VVSE) strains represent to public health is not known due to problems related to its isolation and/or identification from environmental (including water, seafood and healthy

fish tissues) and human samples. To circumvent these problems, we have developed a whole procedure of isolation and identification useful for epidemiological studies and carrier detection.
The isolation protocol is a two-step procedure whose key element is the selective enrichment broth used in the first step, the saline eel-serum broth (ESB) (1). In ESB, the growth yield after 6-8 h of incubation is 1000 times higher for VVSE strains than for putative competitors such as the biotypes 1 and 3 of the species (1).
For VVSE identification, we have developed a multiplex PCR that uses three primer pairs. The first set of primers targets the *V. vulnificus* hemolysin/cytolisin gene (2). The other two were designed after comparing the whole genomes of eel virulent and avirulent strains by suppression subtractive hybridization and confirming their specificity by PCR with a total of 150 isolates belonging to *V. vulnificus* and other species (3).
To select the organ to be sampled for carrier detection in the field, we artificially infected vaccinated and non-vaccinated eels (4) by bath challenge and used the developed protocol to follow the pathogen survival in external and internal organs at different times post-infection. The pathogen was eliminated from all organs in few hours by recently vaccinated eels, whereas it survived on the gills of non-vaccinated eels.
We validated the whole protocol in the field after the isolation of. VVSE strains from seawater, tank water and healthy carriers in the absence of epizootics or outbreaks. These strains were also virulent for mice, the animal model used for human virulence prediction. The VVSE isolates were kept in our collection for further genomic and phenotypic characterization.
In summary, our results demonstrate that VVSE strains are present in the aquatic environment and that only with adequate procedures of isolation and identification their true epidemiological relevance could be revealed.

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D-24

Characterisation of Norwegian *Vibrio anguillarum* serotype O2 by pulsed-field gel electrophoresis

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Recent advances in the field of larval production, regulated wild-fisheries and favourable market prices make farming of marine fish species, in particular Atlantic cod (*Gadus morhua*), increasingly commercially attractive, and in recent years cod farming has expanded considerably in Norway, Iceland and Scotland. Although farmed cod are susceptible to a range of bacterial infections, the most significant mortalities reported in Norwegian aquaculture are those associated with *Vibrio anguillarum*. A typing system for *V. anguillarum* comprising 10 serovars (Sørensen and Larsen 1986) has been subsequently refined to include sub-groups O2a and O2b (Rasmussen 1987; Bolinches et al. 1990). Additional sub-groups may exist (Tiainen et al. 1997). Isolates from diseased cod most commonly belong to serotypes O2a and O2b. In view of the lack of current knowledge of heterogeneity amongst pathogenic *Vibrio anguillarum* serotype O2 in the coastal waters of Norway, this study was initiated to investigate the degree of relatedness between strains isolated from geographically distinct areas, from various species of fish, and to assess the suitability of Pulsed Field Gel Electrophoresis (PFGE) as an epidemiological tool for study of vibriosis outbreaks in cod.

A total of 75 Norwegian *V. anguillarum* isolates belonging to serotypes O2a and O2b were subjected to PFGE following cutting with the restriction enzyme *Not*I. The studied isolates originated mainly from diseased farmed cod, although isolates from various gadid fish and other species were included. Icelandic, Danish and Swedish isolates were also included for comparative purposes. We found that restriction cutting with *Not*I produced, in the majority of tested isolates, a PFGE pattern comprising 20-25 bands, 10-12 of which were 99 kb or greater. While clonality was clearly identified in Norwegian serotype O2a within individual outbreaks, there was a generally low degree of inter-outbreak relatedness. In contrast, O2b isolates from Norway and Iceland were found to be closely related. The Swedish O2b strain (isolated from turbot) did not, however, group closely with Norwegian and Icelandic O2b isolates.

Comparatively few O2b isolates have been isolated outside the family Gadidae, more specifically Atlantic cod, coalfish, and haddock, which may indicate a specific host/pathogen relationship. The absence of disease caused by *V. anguillarum* serotype O2-beta in farmed salmonids, combined with the frequent isolation of very closely related strains in farmed cod in Norway supports this view.

In summary, the evidence suggests that *V. anguillarum* O2b isolates pathogenic to cod in Norwegian and Icelandic aquaculture can be split into two clades, the majority of isolates belonging to a single clade of common clonal origin. No specific relationship was identified between any particular PFGE group and geographical area, date of isolation or host species. Nevertheless, a degree of heterogeneity exists which may be of use in epidemiological studies. Serotype O2a isolates also separated into two clades, are generally more heterogenic but show clonality within outbreaks. PFGE should therefore allow epidemiological study in these cases.

Despite vaccination, vibriosis remains a fairly common occurrence in cod farming, and while serotype O2b continue to dominate isolations from diseased cod in Norway, the proportion of serotype O2a isolates appears to be increasing (personal observation). This may relate to the development and widespread use of vaccines against both serotypes, whilst reflecting antigenic heterogeneity and low levels of heterologous protection amongst O2a isolates. Our results suggest that for future vaccine

development, members of both clades of serotype O2b should be evaluated for heterologous protection, while comparison of several different strains of serotype O2a may be required.

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D-25

The live cholera vaccine candidates IEM108 and IEM109 can elicit protective immunity and resistance to CTXF infection in animals

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Live oral vaccine should be effective in cholera prevention and control, whereas the cholera toxin genes *ctxAB*, which are carried by the lysogenic filamentous phage CTXΦ, can be horizontally transferred between toxigenic and nontoxigenic strains. Therefore the issue arises that *ctxAB* can be reacquired by the vaccine strains. The gene *rstR* of CTXΦ renders the phage immunity to superinfection of this phage, so it can be used in the live vaccine strain to prevent the re-acquirement of *ctxAB* through CTXΦ infection and lysogenization.

IEM101, a CTXΦ negative El Tor strain, has been evaluated in animal models and human volunteers and showed to be an ideal live oral vaccine candidate. Based on this strain, we constructed the live candidates which also possess the immunity to CTXΦ infection. The El Tor derived *rstR* gene was introduced into IEM101 in a chromosome-plasmid lethal balanced system based on the housekeeping gene *thyA*. The protective gene *ctxB* was also cloned in the same plasmid to construct the candidate strain IEM108. It is showed that IEM108 can elicit both antibacterial and antitoxic immunity in rabbit models. In addition the cloned *rstR* gene rendered IEM108 immune to infection with the marked wide type CTXΦ.

Another candidate, IEM109, was also constructed through the approach of chromosomal integration of *ctxB* and *rstR*, to enhance the stability of the introduced genes in the candidate. A chromosomal fragment containing TLC element, attB of CTXΦ chromosomal integration site, and RTX cluster responsible for the cytotoxic activity for mammalian cells, were deleted from IEM101, and replaced with *rstR* and *ctxB* in the same location. Animal experiments showed that IEM109 developed antitoxic and vibriocidal immune responses and has the characteristics to effectively prevent CTXΦ infection. The construction of such

candidates, which possess the abilities of eliciting protective immunity, and safety to resist El Tor biotype CTXΦ infection, should be the practical approach in the development of cholera vaccine.

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D-26

Effect of temperature on long term survival and induction of the viable but non-culturable state in the coral-associated bacteria *Vibrio shiloi* and *Vibrio tasmaniensis*

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In vibrios, the viable but nonculturable (VBNC) state is generally induced by incubation in seawater at low temperatures (less than 10°C.) We were interested in comparing the induction of the VBNC state in two *Vibrio* spp. associated with disease in tropical and temperate corals. One species, *Vibrio shiloi,* is found in warm water environments (16-30°C) and is associated with coral bleaching in the Mediterranean coral *Oculina patagonica.* The VBNC state has been described as an important factor in the infection cycle (Israely *et al.,* 2001; Sussman *et al.* 2003). We recently isolated a range of vibrios associated with necrotic disease in the octocoral *Eunicella verrucosa* from the temperate waters (7-17°C) off southwest England . For this investigation, we selected *Vibrio tasmaniensis,* which was identified using AFLP fingerprinting. We attempted to induce the VBNC state in both cultures by starving the cells and maintaining in artificial seawater at 4 and 20 ^{o}C. Culturable colony were determined on tryptone soy agar (1.5 % NaCl) at regular intervals. Viable counts were measured microscopically using the LIVE/DEAD baclight bacterial viability kit (Invitrogen). Morphological and membrane changes were also monitored using scanning (SEM) and transmission electron microscopy (TEM). Results obtained after 157 days for *V.tasmaniensis* and 60 days for *V.shiloi,* indicated that low temperature did not cause any significant decrease in culturabilty of either species. On the contrary, an incubation temperature of 20 ^{o}C caused a significant decline in culturable cells of *V.tasmaniensis*, with almost 70 % of the population being VBNC after 157 days. Surprisingly, *V.shiloi* maintained a significant culturable count at all times. Electron microscopy at various stages revealed a transformation of the shapes of cells from rods to rounded ones, together with profuse blebbing, the production of a polymer like substance, and increased membrane roughness. *V.shiloi* cells revealed an increased periplasmic space and membrane curling; these features were absent in *V.tasmaniensis.* The maintenance of viability and culturable counts of *V.shiloi* at low temperatures suggests that it is likely to remain as a source of infection of coral during winter months.

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- Attendee Map - Geographical distribution of VIBRIO2005 participants

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- From our sponsors -

The BCCM™/LMG Bacteria Collection

BCCM™/LMG is one of the partners of the BCCM™ consortium (Belgian Coordinated Collections of Microorganisms) and is hosted by the Ghent University, Laboratory for Microbiology.

Holding

The BCCM™/LMG Bacteria Collection holds over **20,000 bacterial strains**, representing some 2,000 species, subspecies or pathovars, encompassing **plant associated** and **phytopathogenic** bacteria (pseudomonads, xanthomonads, erwiniae, agrobacteria, coryneforms, etc.), bacteria of **medical** or **veterinary** importance (*Arcobacter, Campylobacter, Helicobacter, aeromonads, flavobacteria, bordetellae, enterococci, streptococci*), **marine** bacteria (*vibrionaceae*) and various groups of **biotechnological** interest (such as lactic acid and acetic acid bacteria, N2 fixers, clostridia, bacilli, streptomycetes, agrobacteria, etc.). Most of the commonly used control, test and bioassay strains are incorporated.

A hardcopy catalogue of all strains of vibrionaceae (380 items) available from BCCM/LMG can be obtained at the conference or can be forwarded on request.

Services

The BCCM/LMG Bacteria collection offers a number of **services** such as:
supply of strains; deposit of strains either for public access or for back-up purposes; deposit of patent strains under the Budapest Treaty; isolation and purification of cultures; characterization, identification and finetyping of bacteria; contract-research; training.
The following **techniques** are applied:

- DNA sequencing and sequence analysis of diverse phylogenetic markers
- DNA: DNA hybridisations
- measurement of the mol % GC of genomic DNA
- genomic fingerprinting: AFLP, rep-PCR, RAPD, PFGE, DGGE and C-sequencing via capillary electrophoresis.
- SDS-PAGE profiling of whole cell proteins
- gaschromatographic analysis of methylated fatty acids (FAME)
- other phenotypic analyses
- computer assisted comparison of the data generated and construction of databases

An identification system based on AFLP DNA fingerprints has been developed at BCCM/LMG specifically for vibrionaceae and is available on a service basis.

The BCCM™/LMG Bacteria Collection

Research interests

Scope of research projects developed by the collection staff and research groups of the host laboratory:

- Microbial diversity in a wide range of environments, polyphasic taxonomy (using complementary molecular characterisation) and identification of various bacterial groups
- Food bacteriology (e.g. lactic acid bacteria, probiotics, food spoilers)
- Antibiotic acquired resistance, epidemiology of clinical isolates from man or animal
- Microbial ecology, symbiotic systems, natural soil bacteria, distribution of genes
- Phytobacteriology and bacteriological aspects of sustainable agriculture, agroforestry and aquaculture
- Biodeterioration (e.g. works of art)
- Bioremediation (e.g. removal of phosphate or heavy metals)
- Bioprospecting and screening
- Normalisation of the certification, distribution and use of bacterial test, control or bioassay strains.

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