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"Giuseppe De Lorenzo"

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"Influence of environmental stress on virulence gene expression in *Vibrio anguillarum*"

Candidato:
Dott.ssa Francesca Crisafi

Tutor: Dott. Vincenzo Saggiomo
Co-Tutor: Dott.ssa Renata Denaro
Dott.ssa Lucrezia Genovese

Il Coordinatore del Dottorato: Prof. Alberto Incoronato

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Chapter 1

General Introduction
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1. Introduction

1.1 Environmental stress and pathogens.

With the word “Stress” is indicated the disturbance of the normal function of a biological system by any environmental factor and in turn the same factors can be identified by their effects on the biological systems.

An organism’s survival from moment to moment depends, at least in part, on its ability to sense and respond to changes in its environment. Mechanisms for responding to environmental changes are universally present in living beings. For example, when mammals perceive a sudden environmental change as threatening, a rush of adrenaline precipitates the well-known “fight or flight” response.

Such physiological stress responses in complex organisms require appropriately regulated interactions among numerous organ systems.

But how do single-celled organisms respond to potentially lethal threats? The hope is that identifying specific mechanisms that contribute to microbial survival under rapidly changing conditions will provide insight into stress response systems across life forms. Bacteria and especially those capable of persisting in diverse environments, such as *Escherichia coli*, provide particularly valuable models for exploring how single-celled organisms respond to environmental stresses.

For example, most bacteria associated with food borne infections (e.g., some *E. coli* serotypes, *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*) can survive under diverse conditions, both inside and outside of the host. To ultimately cause human infection, a foodborne pathogen must first survive transit in food or water, following ingestion, the bacterium must survive to the exposure to some protection strategies against pathogenic microbes, including gastric acid (ranging

from [pH 2.5–4.5], largely depending on feeding status), bile salts (as well as in fish), and organic acids within the gastrointestinal tract. To survive these extreme and rapidly changing conditions, bacteria must sense the changes and then respond with appropriate alterations in gene expression and protein activity. Therefore, one important scientific challenge is to identify mechanisms that control the switch or switches that allow free-living bacteria to adjust to and invade a host organism.

As well as foodborn pathogens, bacterial pathogens, in general, have evolved highly sophisticated mechanisms for sensing external conditions and respond by altering the pattern of gene expression with activation of a set of genes whose products assist in survival and turning off synthesis of functions that are not necessary in a particular environment. These sensor-activator systems allow bacteria to monitor environmental parameters that distinguish host from external environment and adjust gene expression accordingly, particularly by induction of virulence factors (Albright *et al.* 1989; Parkinson and Kofoid 1992). The expression of virulence genes is controlled by regulatory systems in such a manner that the virulence factors are expressed at different stages of the infection process dictated by the changing micro-environment of the host as a consequence of the patho-physiology of infection. Moreover, the environmental control of regulatory mechanisms is mediated by complex processes at the level of transcription and translation. Accordingly, mutations in some of the regulatory systems attenuate virulence of several bacterial species (Dorman *et al.* 1989).

Studies on this topic provide powerful new insights into the field of microbial physiology by enabling identification of genes that may appear to be unrelated in function, but that must be coordinately regulated to enable an organism to survive

and respond appropriately under rapidly changing environmental conditions, such as those encountered by a bacterial pathogen during the infection process. These coordinately regulated genes ultimately may prove to be appropriate targets for development of novel antimicrobial strategies, thus providing tangible realization of the promise and power of the application of genomics tools for improving human health (Boor 2006; Chowdhury *et al.* 1996).

Virulence determinants include all those factors contributing to infection as well as to disease, with the exception of "housekeeping" functions that are required for efficient multiplication on non living substrates. While some determinants fit comfortably into such a scheme (e.g., adherence to host tissues, production of host-specific toxins, invasion into host cells, and resistance to host defense mechanisms), others are in a gray area bordering on housekeeping functions. Thus, bacterial factors that facilitate the acquisition of iron in the host are arguably virulence factors given the prodigious effort the host makes to withhold this critical nutrient from invading microbes (Crichton and Charloteaux-Wauters 1987). However, conditions can be established in vitro under which the same virulence factors are required for growth on, for example, iron-limiting laboratory media. Likewise, the fact that mutations in genes encoding the enzymes of aromatic compound biosynthesis render many bacteria attenuated could qualify these enzymes as virulence factors. Such mutational studies are also of profound interest in understanding the metabolism of microbes during disease and as a practical approach to vaccine development. Interestingly, the coordinate regulation of a potential housekeeping function with a clear-cut virulence determinant can help support the function's role as virulence factor (e.g., regulation by iron of both siderophore biosynthesis and cytotoxin production in *Corynebacterium diphtheriae* or

Escherichia coli). Thus, understanding the regulation of virulence properties can help us define what constitutes a potential virulence factor and indeed can facilitate the identification of new virulence factors on the basis of only their regulatory properties (Knapp *et al.* 1988; Maurelli 1989; Miller *et al.* 1991; Mulder *et al.* 1989; Peterson and Mekalanos 1988).

The multifaceted nature of the host-parasite interaction indicates that more than one virulence determinant is typically involved in pathogenesis. This prediction has been supported by numerous studies showing that specific virulence determinants (e.g., adhesins, invasins, toxins, capsules, etc.) contribute to unique steps in the patho-biology of microbes. These studies have further established that the expression of dissimilar virulence determinants is frequently coordinately controlled by a common regulatory system. Table 1 shows a partial list of the organisms that have virulence factors coordinately regulated by the same environmental signal(s). In virtually all of these examples, this coordinate regulation has as its basis a common regulatory system which controls the expression of genes encoding these virulence determinants. As discussed below, the molecular level of this control is usually transcriptional, but more than one DNA-binding regulatory protein can be involved (Mekalanos 1992).

Table 1. Environmental signals controlling the expression of coordinately regulated virulence determinants in bacteria.

Organism(s)	Environmental signal(s)	Reference(s)
Agrobacterium tumefaciens	Phenolic compounds, monisaccharides, pH, phosphate	Ankenbauer 1990, Binns 1988, Cangelosi 1990, Winans 1990.
Bacillus anthracis	CO^2	Bartkus 1989.
Bordetella pertussis	Temperature, SO^4, nicotinic acid	Janzon 1990, Knapp 1988, Melton 1989, Roy 1991.
Corynebacterium diphtheriae	Iron	Boyd 1990, Pappenheiner 1936, Schmitt 1991.
Escherichia coli	Iron, temperature, carbon source	Bagg 1987, Calderwood 1987, Goransson 1989 and 1990, Tardat 1991.
Listeria monocytogenes	Heat shock	Leimeister-Wachter 1991, Sokolovic 1989.
Pseudomonas aeruginosa	Iron, osmolarity	DiRita 1989 and 1991, Storey 1990.
Salmonella typhimurium	Osmolarity, starvation, stress, pH, growth phase	Buchmeier 1990, Fields 1989, Galan 1990, Miller 1991.
Shigella species	Temperature	Alder 1989, Hale 1991, Maurelli 1989 and 1988.
Staphylococcus aureus	Growth phase	Janzon 1990.
Vibrio cholerae	Osmolarity, pH, temperature, amino acid, CO^2, iron	DiRita 1991, Goldberg 1990, Miller 1988, Peterson 1988, Shimamura 1985.
Yersinia species	Temperature, Ca^{2+}	Barve 1990, Bergman 1991, Cornelis 1989, Fox 1991.

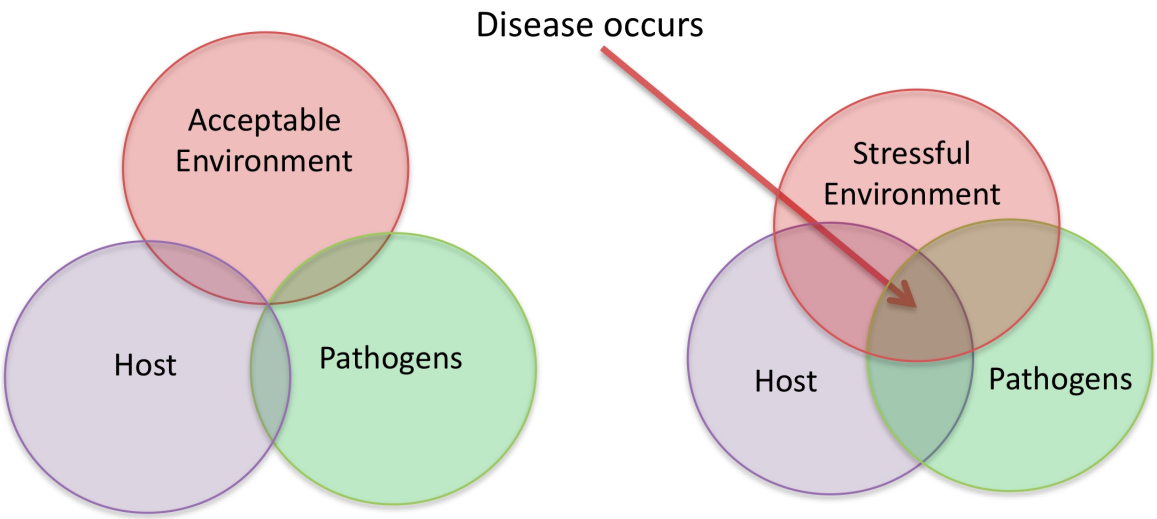
Da **Environmental signals controlling expression of virulence determinants in bacteria.** J J Mekalanos. *J Bacteriol.* 1992 January; 174(1)**: 1–7.**

1.2 Marine pathogens

Bacterial survival in the marine environmental is challenging, due to the large variations in water parameters such as pH, temperature, salinity, nutrient levels, oxygen levels, the amount of sunlight and water current change with time (hour, day, season), and with space (open water, costal water, estuaries) (Thompson *et al.* 2004), this is particularly true for the marine pathogens that have to survive in host or out of the host environment

Bacterial diseases of marine fish come in bewildering array, and the accretion rate of new literature about them has accelerated enormously in the past 2 decades, largely because of urgent problems in marine aquaculture and occasional epizootics in natural population. Bacteria associated with fish can be categorized as primary pathogens; secondary invaders, often with proteolytic abilities, that may be pathogenic for hosts with preexisting infections of other kinds; proteolytic heterotrophs, which invade dying animals and which, if cultured and injected experimentally in massive quantities, may kill some experimental host; or normal marine flora, which may occur on body surfaces of the host, but are not usually pathogenic. Many of the bacteria present in seawater, or on surfaces of fish, can invade and cause pathological effects in fish are injured or subjected to other severe environmental stressors.

Disease is usually the outcome of an interaction between the host (=fish), the disease-causing situation (=pathogen) and external stressor(s) (=unsuitable changes in the environment; poor hygiene; stress). Before the occurrence of clinical signs of disease, there may be demonstrable damage to\weakening of the host.



Conoroy (1984), described a system of useful major groupings which contain most of the important bacterial pathogens of marine fish. They are:

- Gram-negative organisms (Vibrio, Pseudomonas, Aeromonas, Pasteurella) which cause bacterial hemorrhagic septicemias,
- Acid-fast bacteria (Mycobacterium, Nocardia) which cause tuberculosis and nocardiosis,
- Gram-positive pathogens (Streptococcus sp. And Renibacterium salmoninarum) which cause focal or systemic infections and mortality,
- Anaerobic bacteria (Eubacterium) which cause systemic infections and mortality,
- Myxobacteria (flexibacter) which cause gill and fin pathology, and erosion of skin and cartilage.

Of the five groups, gram-negative bacteria, causing hemorrhagic septicemias, are by far the most important fish pathogens, with effects on host populations probably exceeding the combined effects of the other four groups. The groupings will be discussed in descending order of importance to marine fish populations. (Sindermann 1990).

A large group of marine pathogens known belong to the gamma-proteobacteria and within these, the genus Vibrio shows a predominant fraction.

At the genus Vibrio are associated 11 human pathogens including *V. cholerae* etiological agents of epidemic cholera, and the hazardous seafood poisoning agents *V. vulnificus* and *V*. *parahaemolyticus.*

Therefore, many more vibrios are associated with diseases in marine animal, an example is *Vibrio anguillarum* the etiological agents of the vibriosis, a diseases that is largely distributed in marine environmental and in particular in aquaculture.

1.3 Response to stress conditions in Vibrio species.

Temperature is a critical factor affecting pathogenic bacteria. For example, chemotaxis is important for virulence in *Vibrio anguillarum* and it is strongly affected by temperature.

V. anguillarum is most robustly chemotactic at 25°C, and the chemotactic response diminishes in both cooler (5°C, 15°C) and warmer (37°C) conditions (Larsen *et al*. 2004). Increasing temperature may be due to alterations of DNA superhelicity (Dorman 1991). Moreover, in contrast to other pathogenic organisms the virulence genes in *V. cholerae*, are optimally expressed at 30°C and reduced at 37°C (Isberg *et al.* 1988; Parsot and Mekalanos 1990; Pierson and Falkow 1990); it has been proposed that the divergent transcription of a *htpG-like* heat shock gene in *V. cholerae* leads to a proportionate decrease in the expression of *toxR*, coding for a trans membrane DNA-binding protein that positively regulates transcription of the genes for cholera toxin and other virulence determinants (Parsot and Mekalanos 1990). ToxR represses several gene functions which presumably include some necessary for

chemotaxis and motility (Miller and Mekalanos 1988; Strauss 1995). Since motility is involved in establishing the infection, it is possible that at an early stage of the infection process, *toxR* remains repressed and once the bacterium reaches the site of colonization, as yet unidentified environmental signal(s) at the surface of the mucosal epithelium may activate ToxR leading to the expression of virulence genes.

Temperature influences virulence factors also in *V. vulnificus*. An important virulence factor in *V. vulnificus* is capsular polysaccharide (CPS); CPS production appears to be controlled by a phase variation mechanism that can be detected by examining colony phenotype. Encapsulated cells make opaque colonies, while *cps*- cells make translucent colonies. Conversion from CPS+ to *cps*- (from opaque to translucent) is affected by temperature, as increasing the temperature from 23°C to 37°C increased switching for several different isolates (Hilton *et al*, 2006).

Recently, molecular mechanisms of survival in the face of pH stress have been studied intensively in the species *V. vulnificus*. The alternative sigma factor encoded by the *rpoS* gene is required for *V. vulnificus* to survive acid stress (pH <5) in both stationary and exponential phases of microbial growth (Hulsmann *et al*. 2003; Park *et al*., 2004). Other regulatory proteins such as CadC (accessory protein for the Cd^{2+} efflux ATPase CadA), SoxR (superoxide response regulator), and Fur (ferric uptake regulator protein) are also needed for survival of acid stress. The CadC regulator in *V. vulnificus* induces *cadAB* expression, leading to the production of CadA (lysine decarboxylase) and CadB (lysine-cadaverine antiporter). Lysine decarboxylation is one step toward the production of cadaverine, which accumulates in the extracellular space during an acid stress response. The AphB transcription factor also enhances expression of *cadAB* under stressful acidic conditions, by directly activating a promoter that drives

CadC production (Rhee *et al.* 2002; 2006).

Nutrition stress in marine environments is a very common condition. Ninety-five percent of the open ocean is oligotrophic, averaging a scant 50 g of carbon fixed per square meter per year by primary productivity (Atlas and Bartha, 1998). Host organisms, however, are nutrient rich. As vibrios experience transient free-living and host-associated life cycles, these microbes encounter feast or famine conditions in which they are either host-associated (feast) or living in the water column or sand (famine). They must therefore undergo long intervals with little or no growth and metabolic dormancy in their free-living state, followed by brief periods of rapid growth during symbiosis (McDougald and Kjelleberg, 2006). Given this natural history, it is no surprise that many *Vibrio* species possess extraordinarily quick generation times during periods of high nutrient availability, enabling them to out-compete and outgrow other microbial species (Eilers *et al*., 2000; Giovannoni and Rappe, 2000). A study of *V. anguillarum*, demonstrated a link between nutritional stress and virulence. Chemotaxis is an essential activity during infection, and starving (through incubation in phosphate-buffered saline) and *V. anguillarum* cells remained virulent as exponential-phase cells after 2 days, and were still chemotactic post 8 days starvation using LD50 (Larsen *et al*. 2004).

Moreover, marine pathogens live in environments that vary in salinity, and therefore experience high (hyperosmolar) and low (hypoosmolar) osmotic stress. During hypoosmolarity, the obstacles to cellular homeostasis are maintaining appropriate cytoplasmic concentrations of metabolites and ions, preventing cell lysis, and preserving ionic strength and ph (Bartlett 2006). During hypoosmotic shock, some vibrios may increase putrescine content to compensate for decreased K^+ that are

necessary to stabilize the phosphate backbones of nucleic acids. Hyperosmolarity, however, promotes dehydration and shriveling of cells. Microorganisms must be able to import or synthesize counter balancing solutes that are compatible with metabolic and physiological functions. K^+ uptake is frequently stimulated to compensate for the increased external osmolarity. However, negative counter-ions (e.g., glutamate) must also be concurrently imported into the cell or synthesized de novo to sustain the same intracellular net charge (Sleator and Hill 2001). Alternatively, cells can forgo K^+ uptake and import or synthesize neutral compatible solutes, as they carry no charge. Ectoine is such an example and its biosynthesis may be unique to the genus *Vibrio* (Bartlett 2006). *V. fischeri* is also known to possess the ability to synthesize the disaccharide trehalose, which is also a neutral compatible solute for high osmolar stress. Incorporating polyunsaturated fatty acids in the cell membrane may also alleviate vibrios of excess toxic Na^+ by allowing their departure through the more fluid membrane (Valentine and Valentine 2004). Osmotic stress has also been observed to have effects in other vibrios. For example, in the fish pathogen *V. anguillarum*, chemotactic responses to serine are decreased by high osmolarity (≥1.8% NaCl) relative to optimal osmolarity conditions (0.8% NaCl) (Larsen *et al*, 2004).

Proteomic analyses have been completed of *V. alginolyticus* and *V. parahaemolyticus* at different NaCl concentrations to examine resultant changes in gene expression through these physiological shifts (Xu *et al*, 2004; 2005). Since marine pathogens constantly face changes in osmolarity as they shift between marine waters and their native hosts outer membranes proteins are selected to accommodate such changes. Outer membrane proteins OmpW, OmpV, and OmpTolC were discovered to be responsive osmotic stress proteins in *V. alginolyticus* (Xu *et al*. 2005). *ompV* was

expressed at low NaCl concentrations, but not at higher concentrations. Conversely, *ompW* and *ompTolC* displayed reverse changes, being expressed at high NaCl concentrations and down-regulated at low NaCl levels. Interestingly, differential expression of outer membrane proteins, has been suggested by several researchers to play significant roles in symbiosis, including immunogenicity and virulence (Jones and Nishiguchi 2006; Xu *et al*. 2005). Not only were OmpW and OmpV identified in *V. parahaemolyticus* osmoregulation, but elongation factor TU and polar flagellin were implicated as well (Xu *et al*. 2004). Elongation factor TU and polar flagellin were respectively down-regulated and up-regulated at higher salinities, while OmpW and OmpV showed analogous patterns of expression, as in *V. alginolyticus*.

1.4 *Vibrio anguillarum*.

Vibrio anguillarum is an important infectious agent causing *vibriosis* in wild and farmed fish and shellfish. It constitutes part of the normal flora of fish and is associated with rotifer and plankton in the aquatic environmental. Plankton is often part of the food source for fish larvae in aquaculture and therefore, plankton can be the vector for the disease.

Vibrio anguillarum was for the first time described by Bergman in 1909 as the etiological agents of the "red pest of eels" in the Baltic sea. Before this report, Canestrini (1893) described epizootics in migranting eels (*Anguilla vulgaris*) dating back to 1817 that implicated a bacterium named *Bacillus anguillarum*. The pathology of the disease and the characteristics of the bacterium in these two reports suggested that the etiological agents were the same.

V. anguillarum is a polarly flagellated, Gram-negative, curved rod, facultative anaerobe

with a guanine plus cytosine (G+C) content of 43-46%. It grows rapidly at 25-30°C in rich media, such as brain heart, trypticase soy broth or agar containing 1,5% sodium chloride (NaCl). On solid medium, it produces circular, cream-coloured colonies.

List of the phenotypic properties, as well as the salt and temperature range for *V. anguillarum*, has been published (Schiewe *et al.* 1981); it has been recovered sporadically at low temperature from 1°C to 4°C (Olafson *et al.* 1981). Although it is a halophile, it has been recovered sporadically from freshwater (West and Lee 1982) and there are reports of infections occurring in freshwater (Rucker 1959) while Hoff (1989) has shown that it is able to survive in seawater for more than 50 months.

Currently 23 serotypes (O1–O23) are distinguished based on the European serotyping system. Only serotypes O1, O2, and O3 encompass pathogenic strains. Strains belonging to serotype O1 are mainly responsible for *Vibriosis* outbreaks. Serotype O2 has less impact and occasionally.

The mode of infection in fish consists of three basic steps (Alsina and Blanch 1994; Larsen *et al.* 2001; Larsen and Boesen 2001): (i) the bacterium penetrates the host tissues by means of chemotactic motility; (ii) within the host tissues the bacterium deploys iron-sequestering systems, e.g., siderophores, to “steal” iron from the host; and (iii) the bacterium eventually damages the fish by means of extracellular products, e.g., hemolysins and proteases. Grisez *et al.* (1996) showed that infection of turbot (*Scophthalmus maximus*) larvae by *V. anguillarum* occurs in the intestinal epithelium, where the pathogen invades the bloodstream and spreads to different organs, culminating in death of the fish. More recently, Ringo *et al.* (2001) detected bacterial endocytosis in the pyloric ceca and midgut of arctic charr (*Salvelinus alpinus L.*) adults and suggested that the whole gastrointestinal tract of fish may be subject to

infection. Internal symptoms of disease in fish caused by strains of vibrios include intestinal necrosis, anemia, ascitic fluid, petechial hemorrhages in the muscle wall, liquid in the air bladder, hemorrhaging and/or bloody exudate in the peritoneum, swollen intestine, hemorrhaging in or on internal organs, and pale mottled liver (Austin and Austin 1999). External symptoms include sluggish behavior, twirling, spiral or erratic movement, lethargy, darsene pigment, eye damage/exophthalmia, hemorrhaging in the mouth, gill damage, white and/or dark nodules on the gills and/or skin, fin rot, hemorrhaging at the base of the fins, distended abdomen, hemorrhaging on the surfaces and muscles, ulcers, and hemorrhaging around the vent. (Thompson 2004).

Water quality, water temperature, presence of a pathogenic serotype and the health and stress status of the animal are crucial for disease onset. Aquacultures are prone to infectious disease outbreaks, because the intimate contact between the animals causes stress and supports the quick spread of vibriosis. Consequently, an entire population is quickly wiped out or at least substantially damaged. Once introduced into a fish farm, *V. anguillarum* persists for at least two years and total eradication is virtually impossible (Pedersen and Larsen, 1998; Austin and Austin, 1999; Thompson *et al.* 2006).

In aquaculture settings, *Vibrio anguillarum* is controlled by the use of antibiotics and vaccination. Antibiotics are indirectly administered to the fish through the water or the feed. Drugs like ampicillin chloramphenicol, sulfonamides and trimethoprim have been used extensively. Consequently, the number of drug resistant strains has increased inside the fish farms and in the surrounding environment.

Vibrios have a high level of genome plasticity and the aquatic environment provides a

large pool of mobile genetic elements, and both properties aid in the evolution of multi-drug resistant strains (Pedersen *et al*. 1995). Molecular and genetic analysis shows that the genes encoding antibiotic resistance were often found in plasmids many of them conjugative (Aoki *et al.* 1984; Toranzo *et al.* 1984).

Vaccines are administered by bathing the animals in a vaccine solution or intraperitoneal injection (Ketcheson *et al.* 1980; Toranzo *et al.* 1997). Traditional vaccines consist of formalin-killed *V. anguillarum* or membrane components (Agius *et al* 1983). An alternative to the antibiotics and vaccines is the use of beneficial bacteria or probiotics. Lactic acid bacteria, *Baciliales*, *Flavobacteria* and others are commonly used as probiotics. They are able to produces metabolites that inhibit colonization or growth of harmful bacteria and they compete with potential pathogens for space and nutrients (Weber 2010). Presence of probiotics improves water quality and thereby, the living condition the living conditions for the animals (Versecueere *et al.* 2000; Boor *et al.* 2006).

1.5 *V. anguillarum* virulence factors.

Several virulence factors have been identified in *V. anguillarum*, but the majority of them are only partially characterized and their precise role in virulence remains to be answered.

One virulence factor of *Vibrio anguillarum* is a very efficient iron sequencing system; *V. anguillarum* serotype O1 produce anguibactin, which is encoded on the virulence plasmid pJM1 and serotype O2 strains produce vanchrobactin, which is encoded on the chromosome.

Iron is an essential element for nearly all microorganisms, and bacteria have evolved

systems, such as siderophores, to scavenge ferric iron from the environmental and in particular from the iron-binding proteins of their hosts (Braun and Killmann 1999; Bullen and Griffiths 1999: Di Lorenzo *et al.* 2004).

V. anguillarum serotype O1 produces a very efficient iron sequestering system, encoded by the 65-kilobase pair (Kb) pJM1 plasmid, (Crosa *et al.* 1989; Tolmasky and Crosa 1990; 1991) This iron sequestering system utilies the siderophore anguibactin (Crosa 1980), which is synthesized from 2,3-dihydroxybenzoic acid, L-cysteine and N-hydroxyhistamine (Di Lorenzo *et al.,* 2003; López and Crosa, 2007). This iron sequestering system consists of the siderophore anguibactin, its biosynthesis genes, and genes for the receptor complex. The biosynthesis genes (*angB/G, angM, angN, angR, and angT*) are located on both, the chromosome and the virulence plasmid, but the genes encoding for the receptor complex (*fatA, fatB, fatC, and fatD*) are located on the virulence plasmid (Actis *et al.* 1986; Stork *et al.* 2002). When free available iron is low, anguibactin is excreted outside the cell and competes for iron with the high-affinity iron-binding proteins present in the fish. The Fe (III)-anguibactin complex is specifically recognized by the membrane receptor FatA (outer membrane receptor) and then, FatB, a lipoprotein anchored to the inner membrane and protruding into the periplasm, binds the complex and delivers it to FatC and FatD which are inner membrane permeases that transport the ferric-anguibactin complex to the cytoplasm using energy derived from ATP hydrolisis. FatA requires energy derived from the proton-motive force across the cytoplasmic membrane to internalize the ferric-anguibactin (Weber 2010). In *Vibrio anguillarum* the TonB2 system is required for ferric-anguibactin transport (Stork *et al.* 2004). The TonB2 protein, anchored to the cytoplasmic membrane enables FatA to utilize the energy from the Proton Motif

Force. Another protein, TtpC that is highly conserved in pathogenic Vibrios, is encoded in the *tonB2* gene cluster. TtpC is crucial for the iron transport mediated by the TonB2 system (Stork *et al.* 2007).

Expression of the iron sequestering system is regulated by the concentration of iron inside the cell via at least 4 regulators, three plasmid-encoded (AngR, TAF, RNAβ) and one chromosomal (Fur) (Stork *et al.* 2002). In high iron concentration, expression of this iron-transport system is transcriptionally repressed by Fur when the protein is bound to ferrous iron (Tolmasky *et al.* 1994) and post-transcriptionally repressed by the antisense RNA RNAβ, which decreases gene expression by controlling termination at an intergenic region within an essential operon of the iron-uptake system (Chai *et al.* 1998; Stork *et al.* 2002). The system is further controlled by two positive regulators TAF (transacting factor) and AngR (anguibactin system regulator) that likely activate the system as a protein complex (Chen *et al.* 1996; Salinas *et al.* 1989; Wertheimer *et al.* 1999). AngR is repressed by both Fur and RNAβ under high iron conditions. The repression is released in iron limiting conditions, allowing AngR together with TAF to induce expression of iron-uptake system (Stork *et al.* 2002). The anguibactin siderophore positively regulates the system by a positive feedback loop (Chai *et al.* 2004; Croxatto 2006).

All seroptype O2 strains and several plasmidless O1 strains produce the siderophore, vanchrobactin. The genes coding for components in the vanchrobactin mediated iron uptake are located on the chromosome. VabA, VabB and VabC are involved in 2,3-dihydroxybenzoic acid synthesis (DHBA), whereas VabE and VabF are required for siderophore assembly. VabS exports and VabH presumably degrades the siderophore (Balado *et al.* 2006). VabD is most likely a PPIase (peptidylprolylisomerase), VabG is a

putative 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and VabR is a LysR transcriptional regulator required for *vabG* expression regulation (Balado *et al.* 2008). FvtA is the receptor for vanchrobactin (Balado *et al.* 2009). Vanchrobactin is also transported via the TonB2 system (Stork *et al.* 2004).

Pathogenic strains of *V. anguillarum* can acquire iron from heme and heme-containing proteins, including hemoglobin and hemoglobin–haptoglobin, by a siderophore independent mechanism (Mazoy and Lemos 1991). It is believed that acquisition of iron from heme is facilitated by the production of hemolysins or cytotoxins that can lyse host cells and release intracellular heme (Garcia *et al.* 1997), and several hemolysin genes have been described in *V. anguillarum* strains to date (Hirono *et al.* 1996; Rodkhum *et al.* 2005; Rock and Nelson 2006; Lemos *et al.* 2007). Uptake is independent of the virulence plasmid pJM1, but both, TonB1 and TonB2 can mediate transport (Stork *et al.* 2004). A gene cluster coding for nine potential proteins involved in heme uptake and utilization have been identified. Among these genes, the operon *huvAZBCD* is essential for uptake and utilization. They encode for a heme receptor complex that contains a receptor (HuvA) and a translocation machinery (HuvZBCD) (Mourino *et al.* 2004). However, the significance of the heme acquisition system in virulence still remains to be answered.

In addiction to causing a fatal hemorrhagic septicemia, vibriosis also causes massive tissue destruction in the fish, indicating that several secreted extracellular products may contribute to these pathologic observations.

Five hemolysins VAH1–5 are present in *V. anguillarum*. VAH1 is related to a hemolysin in *V. cholerae* ElTor and VAH2 has 89% identity to a hemolysin present in *V. vulnificus*. VAH3, VAH4 and VAH5 are similar to *V. cholerae* O1 proteins: a hemolysin-related

protein, a thermostable hemolysin, and a putative hemolysin, respectively (Hirono *et al.* 1996; Rodkhum *et al.* 2005).

The zinc dependent metalloprotease EmpA, a homologue to the hemagglutinin protease HapA of *V. cholerae*, is one of the dominant products found in culture supernatants. The protease of 36 KDa requires Zn^{2+} for activity and Ca^{2+} for stability (Norqvist *et al.* 1990). EmpA is a mucinase and might function similar to HapA (Croxatto *et al.* 2007). HapA activates hemolysin, degrades tight junctions, and is involved in mucin penetration and the detachment of the bacteria from epithelial cells (Silva *et al.* 2003). Expression of *empA* is induced by gastro-intestinal mucus and is also regulated by RpoS during stationary phase and by quorum sensing [Milton *et al.,* 1992; Denkin and Nelson, 1999 and 2004; Staroscik *et al.,* 2005).

Moreover, recently, forty putative virulence related genes were identified by a random sequencing procedure (Rodkhum *et al.* 2006). Among these potentially novel genes, an RTX (repeat toxin) gene cluster, *rtxACHBD* was indentified. The RTX toxin is encoded by *rtxA*, *rtxBDE* encode the RTX toxin transporter (ABC transporters), *rtxC* codes for the RTX toxing activating protein and *rtxH* codes for a conserved hypotetical protein (Li *et al.* 2008).

The ability to penetrate tissue is an important virulence factor for pathogenic bacteria. Attachment requires motility and chemotaxis. *V. anguillarum* posses a single polar flagellum composed of five flagellin proteins FlaA-E, of which FlaA is essential for virulence (Milton *et al.* 1996). The flagellum functions in the virulence of water-borne *V. anguillarum* by mediating chemotactic motility (Actis *et al.* 1999). However, the flagellum and chemotaxis are dispensable once the pathogen is introduced through the fish epithelial layer by injection (Actis *et al*. 1999; Agius *et al.* 1983). This

indicates that chemotaxis is required for virulence at a stage of infection prior to penetration of the fish epithelium.

RpoN, the alternative sigma factor σ54, regulates the expression of flagellar genes and deletion of the *rpoN* gene attenuates virulence. *V. anguillarum* displays a strong chemotactic response towards intestinal and skin mucus, which can be used as a carbon source (Garcia *et al.* 1997). Chemoattractants, like free amino acids or carbohydrates, induce smooth swimming leading the bacteria to the fish epithelia. For successful infection *V. anguillarum* has to sense and swim to the animal and to pass the fish integument. (O´Toole *et al.* 1996, 1997 and 1999; Milton *et al.* 1996; Ormonde *et al.* 2000).

The outer membrane porins have an important role in the bile resistance in *Vibrio anguillarum*; cells produce a 38-kDa major outer membrane porin OmpU, with weak cation selectivity and moderate surface charge. Loss of OmpU results in increased bile sensitivity, but surprisingly not in decreased virulence. Resistance to bile is required to survive and colonize the intestines. Expression of *ompU* is regulated by the transcriptional activator ToxR, a homologue to *V. cholerae* ToxR; the presence of bile salts can also induce *ompU* expression (Wang *et al.* 2002 and 2003).

Outline of this thesis.

Bacterial pathogens thrive in a multitude of environments and are exposed to a vast array of stress conditions in their host and especially outside of the host environment; moreover the environmental parameters have an important role in the dissemination of disease especially in aquaculture farms.

The aim of the research presented in this thesis was the study of the environmental stress influence on the expression of virulence genes in the fish pathogen *Vibrio anguillarum*.

Chapter 2 describes the methodological approaches used in this study distinguished in (i) microbiological approach, (ii) molecular approach and (iii) biochemical approach.

Chapter 3 describes the differential response to environmental stress by seven selected virulence genes in *Vibrio anguillarum*. The environmental conditions tested were, variations of temperature, salinity and concentration of iron which can strongly influence the persistence and dissemination of marine pathogens. The target genes tested are all involved in the process of infection in fish that is articulated in three main steps: (i) the penetration of the host tissues by means of chemotactic motility; (ii) the growth in vivo by means acquisition of iron in the host tissues; and (iii) the dissemination of damage in the fish by means of extracellular products, e.g., hemolysins and proteases. The object of this part of the work was to characterize the differential response in gene expression after the exposition to a specific stress condition in two strains of *Vibrio anguillarum*: *Vibrio anguillarum* 975/I serotype O1, a virulent strain isolated from infected fish that carries the plasmid pJM1 and *Vibrio anguillarum* ATCC43307 serotype O3 an avirulent plasmidless strain. As part of the

studies we evaluated the efficiency and reliability of real time PCR to detect changes in the bacterial gene expression. Measurements were carried out by analyses of subtractive expression pattern comparing quantitative expression of target genes in optimal condition of growth and stressed conditions by real time PCR using the comparative Ct method.

The trends of gene expression were significantly different in the two tested strains when exposed to variation of stress conditions. In general, expression of the *toxR* gene seems to be the most sensitive to the environmental variation. Moreover, the strain ATCC43307 serotype O3 showed an increase in expression of *tonB2* and *fur* independently of the parameter changed. We were able to detect a significant increase in the expression of plasmidic genes in serotype O1 in iron starvation condition and the over expression of *empA* gene in the serotype O1 during the incubation of *Vibrio anguillarum* cells at 15°C.

Chapter 4 describes the influence of temperature, sodium chloride concentration and growth media on the EmpA metallo-protease production in *Vibrio anguillarum* 975/I, deepening the preliminary result collected in the chapter 3. The experimental analysis was carried out following two different approaches; a molecular approach, with the aim to evaluate the influence of the stressors on the expression of *empA* gene and a biochemical approach in order to determine the post-trascriptional control of environmental parameters tested on the EmpA metallo-protease secretion.

The results showed that the expression of *empA* gene is dependent on the temperature confirming the precedent result obtained in the chapter 3. However increase in *empA* expression wasn't correlated with the increase of EmpA metallo-protease secretion. Whereas it is noticeable the influence of growth media or salinity

in the post-transcription control of EmpA; the most significantly production of EmpA metallo-protease was recorded in CM9 at the NaCl concentration of 0,5%.

Chapter 5 describes the application of the real time PCR as a rapid and specific diagnostic method for the diagnosis of *vibriosis* in the sea bass specimens.

In this part of the work was carried out a comparison between the classical microbiological method for the determination of *Vibrio anguillarum* cells in infected tissues and the new molecular approach using real time PCR to detect *16SrDNA*, a constitutive gene, and *toxR* a virulence gene.

The Real Time PCR was carried out by the method of absolute quantification. The results show that the molecular approach used is more rapid and specific in the determination of *Vibrio anguillarum* in target tissues, kidney and liver, of sea basses respect the classical microbiological methods. Moreover both genes used as molecular marker, *16SrDNA* and *toxR*, can be consider good target genes with different sensitivity for the detection of *Vibrio anguillarum* in fish tissues.

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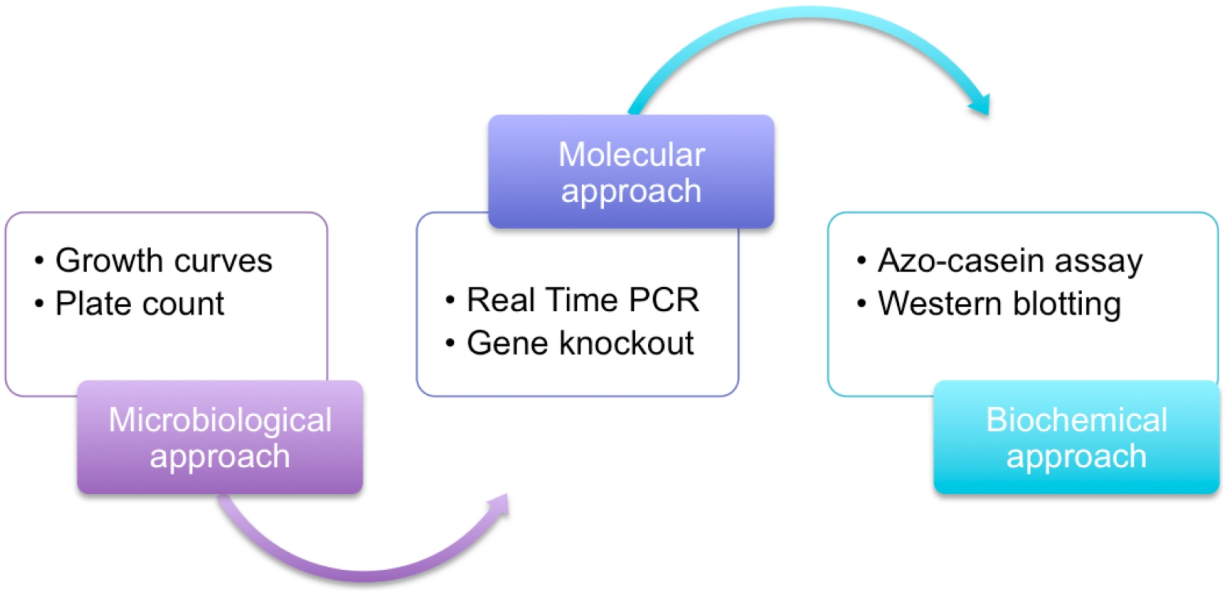
Chapter 2

Experimental Approach

2. Experimental approach



2.1 **Microbiological approach**

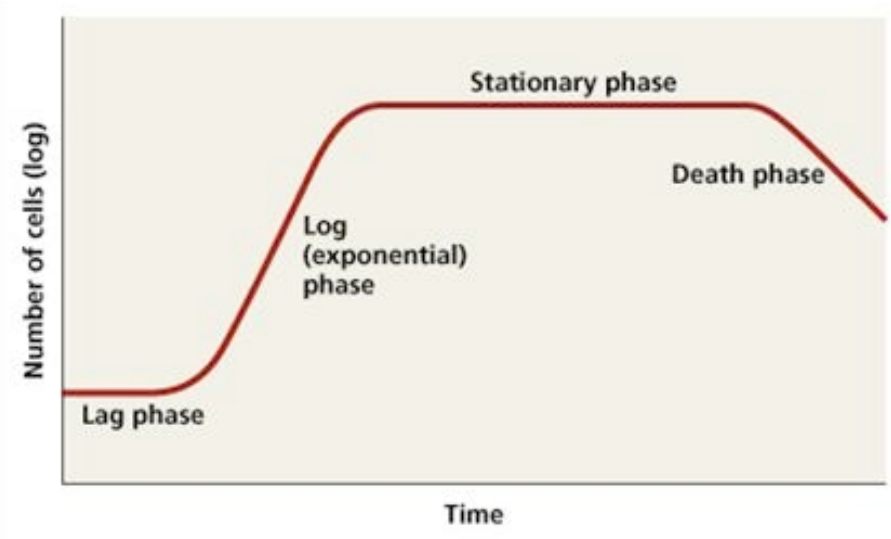
2.1.1 Growth curve and plate count

The classical microbiological techniques were the first step of experimental approach for all chapters of the thesis. Growth curves for different strains and the comparison between growth curves obtained at several stress conditions, were the first evidence of a stress influences on *Vibrio anguillarum*. Several authors suggest that there is a correlation between the growth rate and the environmental stress; Guillier *et al*. (2005) have shown that the lag time distribution is strongly dependent on the stress undergone by the cell; it results in an increased variability of lag times and in their particular distribution.

In the present thesis was studied the effect of stress on the duration of the lag phase and the calculation of the growth rate (Reichert-Schwillinsky *et al.* 2009; Eguchi *et al.* 1996; Guiller *et al.* 2005).

In general, bacteria display a characteristic four-phase pattern of growth in liquid culture:

1. The **Lag phase:** a period of slow growth during which the bacteria are adapting to the conditions in the fresh medium;
2. The **Log Phase:** during which growth is exponential, doubling every replication cycle;
3. **Stationary Phase:** occurs when the nutrients become limiting, and the rate of multiplication equals the rate of death.
4. **Logarithmic Decline Phase** occurs when cells die faster than they are replaced. (This latter occurs over a much longer period of time that the previous three.)



Bacterial population in the culture is estimated by measuring its turbidity, (to which it is proportional) using spectrophotometer. Turbidity is classically measured as the absorbance at 660 nanometers wavelength and indirectly measures all bacteria (cell biomass), dead and alive.

The spectrophotometric values were conformed using the plate count method. The standard plate count method consists of diluting a sample with sterile saline or in growth media until the bacteria are dilute enough to count accurately. That is, the final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons (too few may not be representative of the sample), and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct **colony-forming units (CFUs)**. The assumption is that each viable bacterial cell is separate from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10-4 to 10-10) is normally plated because the exact number of bacteria is usually unknown. Greater accuracy is achieved by plating duplicates or triplicates of each dilution.

The plate count method as experimental approach was applied in the chapter 3, with the aim to detect *Vibrio anguillarum* cells in fish tissues in comparison with the most rapid and specific Real Time PCR method. This comparison was useful to obtain a control of validity of a new molecular approach and a "measure" of efficiency of the method.

2.2 Molecular approach

2.2.1 Real Time PCR.

The real time PCR technique in this research was applied to:

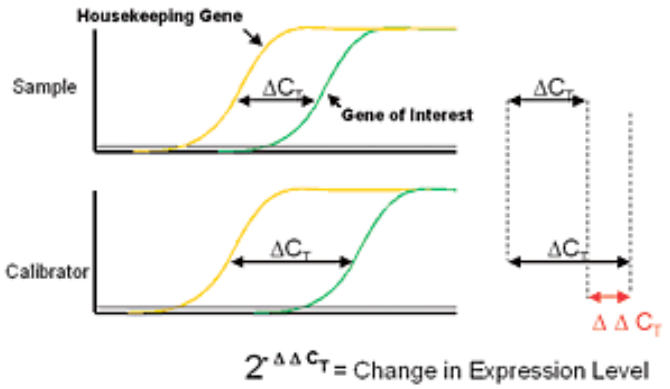
(i) monitor the differential expression of virulence target genes during exposure to different environmental stress conditions; and (ii) detect and quantify *Vibrio*

anguillarum in infected fish. Two variants of RT PCR were carried out: the relative quantification and the absolute quantification.

2.2.3 Real Time PCR relative quantification.

Relative quantification measures the relative change in mRNA expression levels. It determines the changes in steady-state mRNA levels of a gene across multiple conditions and expresses it relative to the levels of an internal control RNA. This reference gene is often a housekeeping gene and it is usually expressed at a fairly constant rate in cells as it subserves some constant physiological requirement.

In this thesis was used the comparative Ct method, also know as $2^{-\Delta\Delta Ct}$ (Pfaffi 2001; Livak and Schmittgen 2001; Mikesova *et al.* 2006; Nicot *et al.* 2005). Comparative Ct method is adequate for investigating physiological changes in gene expression levels. In this work, the comparative method was carried out to test the differential expression of target genes during growth under different stress conditions using the standard condition of growth as calibrator. The amount of target, normalized to an endogenous reference and relative to a calibrator is given by: $2^{-\Delta\Delta Ct}$.



A validation test must be made to determine if the target genes expression are comparable to that of the housekeeping gene. The slopes of the standard curves of

target and reference genes using dilution series of cDNA samples are compared. For this the ΔCt between each point on the dilution series is determined and plotted against the log of sample input. If the slope of the lines are between -3,3 and 3,4 the validation test is valid, this mean that the efficiencies of amplification of the target genes are similar to the efficiency of the endogenous control, the curves are parallels and is possible to continue the experiment.

The mathematical formulas applied to obtain the final value are the following:

1. Calibrator = average Ct $_{\text{target gene}}$ – average Ct $_{\text{housekeeping gene}}$ (optimum growth condition);
2. ΔCt = average Ct $_{\text{target gene}}$ – average Ct $_{\text{housekeeping gene}}$ (stress condition);
3. ΔΔ Ct = Δ Ct- Calibrator.

The ΔΔ Ct value permits the calculation of $2^{-\Delta\Delta Ct}$ where "2" is the fold change in amplicons between cycles assuming 100% efficiency. Therefore there should be a doubling of PCR target amplicon every cycle. The ΔΔCT value represents the normalized cycle change between a sample and the reference. Therefore this equation indicates how many doublings or fold changes of amplicon occur between cycles. This protocol will be applied to each target gene and stress condition chosen using the same housekeeping gene and calibrator for all analysis data.

2.2.2 Real time PCR Absolute quantification.

A standard curve is obtained from a dilution series of control template of known concentration. This is known as "standard curve" or "absolute" quantification. The absolute quantification approach is used to measure the exact level of template in the samples, in our case the exact quantification of *Vibrio anguillarum* cells in infected fish tissues.

Under ideal environmental conditions, healthy looking fish without a clinical sign or lesion can carry pathogens that create serious risks for the spread of contagious diseases in the fish populations. Disease becomes evident only when stressful condition occurs. The rapid and specific detection and quantification of pathogens from carrier fish is essential for effective fish disease control. Usually the diagnosis of the disease is carried out by traditional microbiological techniques, agar cultivation followed by phenotypic and serological properties of the pathogen or histological examination (Bernardet *et al.* 1990; Pazos *et al.* 1996).

Conventional microbiological methods needed to identify these organisms are often limited by the length of time required to complete the assays. Molecular techniques can be used to solve that type of problems and increase sensitivity and specificity of pathogens detection.

Real Time PCR amplifies a specific target sequence in a sample than monitors the amplification progress using fluorescence technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence, thereby enabling quantification; absolute quantification attempts a more ambitious task to measure the actual nucleic acid copy number in a given sample. This requires a sample of known quantity (copy number) of the gene of interest that can be diluted to generate a standard curve. This is an external "absolute" standard. Unknown samples are compared with the standard curve for absolute quantification. The primary limitation to this approach is the necessity of obtaining an independent reliable standard for each gene to be analyzed and then running concurrent standard curves during each assay (Gonzales *et al.* 2003; Gharaibeh *et al.* 2009).

2.2.3 Knockout target genes.

To identify if and what role the genes analyzed by real time PCR play in virulence, they were inactivated by insertional mutagenesis. This technique, called "**gene knockout",** has been used in the past to determine the function. In many case, in fact, the function of a particular gene can fully understood only with the mutation of several virulence genes.

This part of work was carried out in the laboratory of Dr. Tolmasky in Cal State Fullerton, California USA. The goal of this part of research was the characterization of the role of a single *Vibrio anguillarum* virulence gene in resistance to environmental stress.

The technique of gene knockout was carried out on *empA*, involved on the production of metalloprotease.

The method includes the following steps:

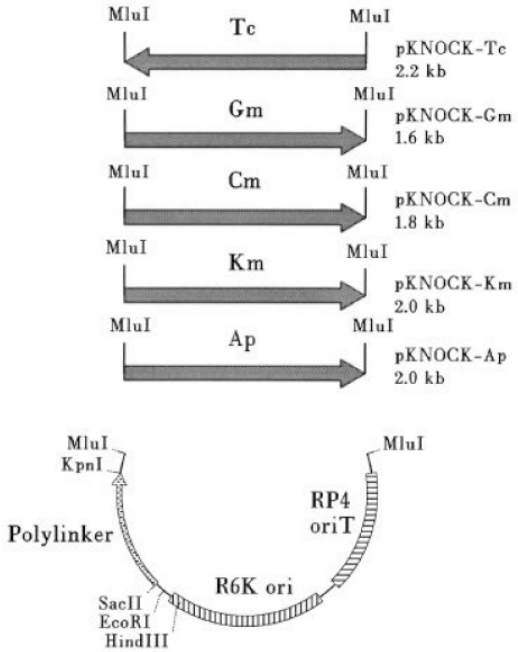
1. Cloning into suicide vector of a target gene fragment truncated at both the 5'- and 3'-end;

2. The mobilization of the hybrid plasmid to the recipient strain;

3. The integrations into the chromosomal copy of the target gene by homologous recombination. The chromosome finally contains two copies of the target gene with the first truncated at the 3'-end and the second truncated at the 5'-end. Both copies are inactive and separate by the vector sequence;

4. The recombinant strains are selected using the appropriate antibiotic.

The vector utilized for the creation of mutant strains was the suicide vector pKNOCK.



The advantage of the pKNOCK, is that it has a polylinker with numerous restriction enzyme sites and can be introduced into the recipient strains via transformation, electroporation or conjugation. The conjugation is used as a very efficient method of DNA introduction into the recipient. Another advantage is that DNA enters the recipient cell as a single strand which is much more effective at homologous recombination when compared to double stranded DNA (transformation and electroporation). Moreover conjugation is the only method that has proven successful for transferring DNA from *E. coli* to *Vibrio anguillarum*.

The pKNOCK plasmid (2.2 kb) also contains a marker gene, in our case the marker was Gm (gentamycin), and an origin of replication, R6K γ-ori. The suicide nature of the plasmid is contributed by the R6K γ-ori, since replication can only occur in *E. coli* strains that have the π protein encoded in *trans*. The mobilization (mob) site is that of the plasmid RP4 (Alexeyev *et al.* 1999).

The first step was the insertion of a fragment of *empA* gene obtained by PCR amplification in the suicide vector. The PCR amplification was carried out using as template DNA extracted from pure culture of *Vibrio anguillarum* 975\I using specific primers for *empA* designed using the total sequence of the gene published on GeneBank (L0258):

EmpAmutF: 241 GGCTTTCAGGTGGTAAAAAGCGTCA

EmpAmutR: 1469 AACTGGTTAGCGACGGTGAACACTTC

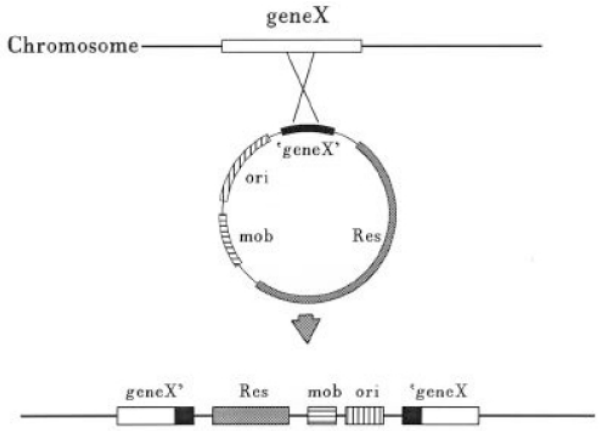
The 1,3 Kb amplicon of was cloned into pCR®2.1 Topo vector (invitrogene) using the TOPO TA Cloning® kit (invitrogene). The vector was introduced in chemically competent cells of *E. coli* TOP10.

Using the QIAGEN Plasmid Mini Kit (Qiagen), the recombinant plasmid was extracted from the cells transformed following the protocol recommended by the supplier. The vector (pCR2.1: *empA*) was digested with *BamHi* and *NotI* restriction enzymes and ligated to the *NotI* and *BamHi* site of the suicide vector pKnock-g.

The newly obtained recombinant plasmid, consisting of pKNOCK-g harboring a fragment of *empA*, was introduced into chemically competent *E.Coli* SM 10 *λ pir* as follows. Three µl of construct and 200 µl of competent cells were mixed and incubated in ice for 30 min after this incubation the mix was heated at 42°C for 90 sec and immediately cooled in ice for 2 min; 800 µl of SOC was added and the mix was incubated for 30 min at 37°C; after the incubation a aliquot 100 µl was plated on LB plates containing gentamycing (25 µg\ml) and kanamycin (50 µl \ml) antibiotics. The plates were incubated at 37°C overnight.

Finally the plasmids were mobilized from *E. coli* SM10 to *Vibrio anguillarum* cells by conjugation. Overnight cultures of *E. coli* SM10 (pKNOCK-g-empA) and *Vibrio*

anguillarum 975\I were cultured on LB and TSA plates, respectively. Cells were scraped from the plates, mixed, and spread on TSA plates with a final concentration of NaCl of 1,5%. The plates were incubated overnight at 25°C. The suicide recombinant plasmid is mobilized from *E. coli* to *V. anguillarum* where it cannot replicate. Therefore, only in those cases where the plasmid integrates into the chromosome by homologous recombination, and therefore the *empA* gene is inactivated, will carry the gentamicin resistance gene.



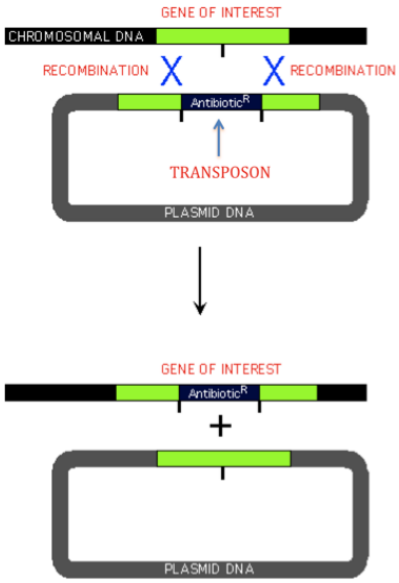
An additional step was added to the standard protocol to enhance the efficiency of mutagenesis. The first part of the new protocol was the same until the creation of pKnock:empA. At this point the additional step consisted of the insertion of a transposon in inside *empA* using the EZ-Tn5™ <TET-1> Insertion Kit (Epicentre - Biotechnologies). Trasposons are mobile DNA sequences found in the genomes of prokaryotes and eukaryotes; the kit can be used to randomly insert the transposon and a tetracycline resistance section marker into target DNA *in vitro*. In our case the target DNA used was pKnock:empA. One aliquot of the reaction was used to transform *E. coli* SM10; the selection of the clones harboring pKnock containing <TET-1> was carried out using plate with the selective antibiotics.

The problem, at this point was to identify the insertion position of transposon in the

vector; to obtain the correct recombination during the following steps of the protocol is necessary that the transposon is inserted inside *empA*. To identify the position of <TET-1>, a randomly selected set of clones were analyzed by PCR amplification using the putative recombinant plasmids (pKnock:empA: <TET-1>) as template. The reactions were carried out using a combination of forward and reverse primers specific for *empA* fragment and <TET-1>.

The clone chosen was used for the successive conjugation with *V.anguillarum* cells.

The protocol was the same described above but in this case the integration of the suicide vector inside the gene is possible by double recombination.



At the end of both procedures, the trans-conjugants were selected and purified on TCBS + NaCl 1,5% plates that contained the selective antibiotics.

After the antibiotics selection on plate were carried out a series of "diagnostic PCRs" using the following primers:

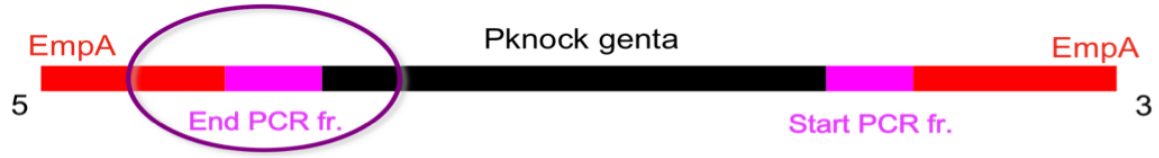
EmpAdiag1F : 5' – CAACGTCAAATGAAGTGGCTATTC- 3'

EmpAdiag1R: 5' – ATTCTCCGTTGGAGGCACTACGCC – 3' designed on the chromosomic portion of *empA* gene and

pKnockdiagnF 5’ – TGCGAATAAGGGACAGTGAAGAAG – 3’

pKncokdiagnR 5’ – CTTCTTCACTGTCCCTTATTCGCA – 3’ designed on the pknock sequence.

The first PCR was carried out using as template DNA extracted from one trans-conjugant and as primers EmpAdiagn1F and of pKnockdiagnR. The fragment amplified was sequenced and from the sequence analysis was identified a clear site of the homologous recombination:



A second PCR analysis was carried out on the same template but using as primers EmpAdiagF and EmpdiagR. The size of amplicon obtained was 1.5Kb corresponding almost with the total size of *empA* gene with out insertion. Also in this case the PCR fragment was sequenced and the sequence obtained correspond perfectly with *empA* gene without interruption:

The result was ambiguous and led us to hypothesize that there were two copies of *empA*: one carrying the insertion and one wild type.

The activity of the gene was analyzed by real time PCR relative quantification using as template cDNA extracted from the “mutant”. The data were analyzed using the *empA* expression in wild type as a calibrator and *rpoN* as a housekeeping gene. The results collected didn’t show any substantial differential expression of *empA* in the “mutant” strain. Moreover a test about the protease activity was carried out; culture supernatants collected from mutant and from wild type were assayed by observing

zones of hydrolysis on 1% casein agar plates containing 2% NaCl. The results were showed that both strains produce similar protease activity.

The protocols applied for the construction of *Vibrio anguillarum empA* mutant require further investigations.

About the discontinuity of the results were advanced several hypothesis:

1. The presence of more copies of *empA* in *Vibrio anguillarum*;
2. Difficult in the selection after conjugation process;
3. Troubles in the integration of suicide vector during the recombination, about this the successive experiments could be carried out using a different suicide vector.

2.3 Biochemical approach

2.3.1 Azo-Casein assay & western blotting.

In order to complete the analysis of the influence of environmental stress on *empA* gene in *Vibrio anguillarum*, in addiction to examining the expression at the transcriptional level was analyzed the effective secretion of protease during the stress exposition.

Two methodologies were applied; the azo-casein assay and the western blot analysis.

The azo-casein assay is a method used to estimate the proteolityc activity in *Vibrio anguillarum* cells. In this work the technique was utilized to detect the protease activity in *Vibrio anguillarum* cells grown under experimental conditions (Engel and Teuber 1998).

The main principle of azo-casein test is the separation of the azo-molecules from the casein protein during the azo-casein degradation by bacterial extracellular proteases.

The azo-molecules released in this reaction have a unique absorption of 442nm. To calculate the Unit of protease activity in necessary to know the Optical Density at 442nm and the CFU present in the samples tested. The mathematical formula used to find the unit of protease activity per ml was the following:

Protease activity Unit = $[1000(OD_{442})/CFU)] \times (10^{9})$

The limitation of this method was the unspecifictiy; the results obtained were, in fact, referred to the total portion of bacterial proteases secreted.

With the aim to test the effective present of the metallo-protease EmpA the western blotting was carried out.

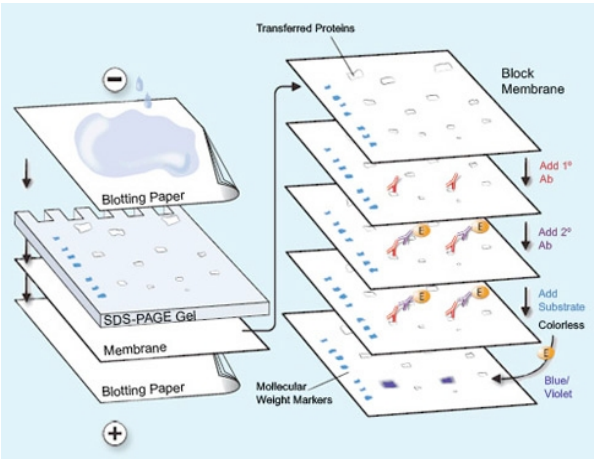
The term “blotting”, in fact, refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin *et al.* in 1979 and is now a routine technique for protein analysis.

The first step in a Western blotting procedure is to separate the macromolecules using gel electrophoresis. Sufficiently separated proteins in an SDS-PAGE can be transferred to a solid membrane for WB analysis. For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the gel and onto the membrane in the same pattern as they separate on the SDS-PAGE. All sites on the membrane, which do not contain blotted protein from the gel can, then be non specifically “blocked” so that antibody (serum) will not non-specifically bind to them, causing a false positive result.

To detect the antigen blotted on the membrane, a primary antibody (serum) is added at an appropriate dilution and incubated with the membrane. If there are any

antibodies present which are detected against one or more of the blotted antigens, those antibodies will bind to the protein(s) while other antibodies will be washed away at the end of the incubation. In order to detect the antibodies which have bound, anti-immunoglobulin antibodies coupled to a reporter group such as the enzyme alkaline phosphatase are added (e.g. goat anti-human IgG- alkaline phosphatase). This anti-Ig-enzyme is commonly called a "second antibody" or "conjugate". Finally after excess second antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody bound to the protein.

The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme produces light as a byproduct. The light output can be captured using film, a CCD camera or a phosphorimager that is designed for chemiluminescent detection. Alternatively, fluorescently tagged antibodies can be used, which are directly detected with the aid to fluorescence imaging system. Whatever system is used the intensity of the signal should correlate with the abundance of the antigen on the membrane.



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Chapter 3

"Screening of virulence gene expression in *Vibrio anguillarum* 975/*I* and *Vibrio anguillarum* ATCC43307 after exposition to environmental stress."

3. Screening of virulence gene expression *Vibrio anguillarum* 975/*I* and *Vibrio anguillarum* ATCC43307 after exposition to environmental stress.

3.1 Introduction

Environmental parameters such as pH, temperature, salinity, nutrients, pollution, presence of antibiotic, iron concentration have been identified as inducers of phenotypic or genetic adaptation in bacteria.

Several studies on genetic of stress adaptation and virulence in *Vibrio cholerae* (Faruque *et al.* 2004, McDougald *et al.* 2003) noticed that bacteria exposed to stress conditions implement adjustments on phenotypic markers. An example of phenotypic adaptation is the production of proteins in response to harmful substances (e.g. bile in the case of pathogens) and the acquisition of resistance for subsequent exposures, as occurs after the exposition to sub-lethal concentrations of antibiotic. Many bacterial pathogens regulate the expression of virulence genes in a co-ordinate manner in response to changes in the environment. For example, the human pathogen, *Vibrio cholerae*, possesses a virulence regulon composed of over 20 genes involved in colonization, toxin production and bacterial survival within the host, which are co-ordinately regulated by external stimuli, such as temperature, pH and osmolarity. Although the expression of the regulon is dependent upon the transcriptional activator ToxR, most of these genes are controlled by a second transcriptional activator, ToxT, which is itself positively regulated by ToxR. The mechanisms by which environmental stimuli influence the ToxR regulon are not yet understood, but ToxR-mediated control over the expression of *toxT* clearly plays a role. The recent finding that the global regulator cAMP-CRP also influences the

expression of the ToxR regulon under various environmental conditions raises new issues regarding the pathways and mechanisms by which this regulation is achieved and indicates that multiple overlapping systems are involved (Skorupski and Taylor 1997). ToxR has recently been found in a number of other *Vibrio* and *Photobacterium* species, including three fish pathogens, of which *V. anguillarum* is one (Lee *et al.* 2000; Li *et al*. 1993; Okuda *et al.* 2001; Osorio *et al.* 2000; Reich *et al.* 1994; Welch and Bartlett 1998); according to Wang (2002) ToxR in *V. anguillarum* may be part of a regulatory cascade that responds to various environmental signals but, until now, there aren't results supporting this hypoteshis.

In particular, *Vibrio anguillarum*, the etiological agent of vibriosis, is considered an opportunistic pathogen, ubiquitous in marine environmental and in microflora of marine fish and it is strongly influenced by environmental parameters (Austin *et al.* 1993; Bolinches *et al.* 1986; Bowser *et al.* 1981; Mizuki *et al*. 2006); for this bacteria the exposition to a particular environmental stress can switche on the pathogenicity causing the development of infection (Sugita *et al.* 2008). Several authors have indentified the temperature, salinity, iron concentration and pollution as environmental stressors that can influence the pathogenicity in *Vibrio anguillarum*, suggesting that the control of this parameters could decrease the incidence of infection in aquaculture farms. (Crosa *et al.* 1980; O'Toole *et al*. 1996; Hauton *et al.* 2000; Chu *et al*. 1996).

In general, such parameters can be perceived by bacteria as environmental changes, to which they respond with adaptive behaviour, (gene expression, activation of starvation mechanisms, protein production) or as a condition which simulate host environment responding with a gene expression profile which involve virulence genes

also.

The aim of this work is to study the influence of environmental parameters on two strains of *Vibrio anguillarum,* 975/I serovar O1(virulent) and ATCC43307 serovar O3(avirulent) on expression of selected genes after exposure to a particular environmental stress.

The genes tested were chosen on the basis of the principal phases of infection included (i) the approach to host, (ii) the growth in vivo and (iii) the damage. In detail the genes studied were:

i. *omp*, coding the Major outer-membrane protein involved in the bile resistance and in the biofilm formation (Wang *et al.* 2003) and *toxR*, involved in the responses to environmental stress (Okuda *et al*. 2001);
ii. *angR* and *fatA*, involved in the iron-uptake system mediated by the pJM1 plasmid (Werheimer *et al* 1999; Waldbeser *et al.* 1993), *fur*, involved in the regulation of the iron uptake sistem (Chai *et al.* 1998) and *tonB2*, involved in the transport of iron inside the cells (Stork *et al.* 2006);
iii. *empA*, coding the metalloprotease secreted during the infection (Denkin *et al.* 2004).

3.2. Materials and Methods

3.2.1 Bacterial strains and Media.

The bacterial strains used in this study were: *Vibrio anguillarum* 975/I belonging to serogroup O1 a local (Mediterranean Sea) virulent strain (kindly provided by Dr. Manfrin, Istituto Zooprofilattico delle Venezie – Italy) isolated from sea bass specimens, and *Vibrio anguillarum* ATCC 43307 belonging to serotype O3 a collection

strain.

The strain 975/I carries the plasmid pJM1 while *Vibrio angillarum* ATCC 43307 hasn't plasmid and it is consider a not virulent strain.

Cells were routinely grown in Tryptic Soy Broth (Pancreatic Digest of Casein 17.0 g\l, Enzymatic Digest of Soybean Meal 3.0 g\l, Sodium Chloride 5.0 g\l, Dipotassium Phosphate 2.5 g\l, Dextrose 2.5 g\l supplemented with 1% of NaCl) and on TCBS plates (Thiosulfate-Citrate-Bile Salts-Sucrose supplemented with 0.5% of NaCl) at 25°C.

3.2.2 Growth and stress condition.

To study the influence of environmental stress on the expression of virulence genes, the bacteria were cultured under experimental conditions. The role of temperature was investigated on cells grown in TSB with the final concentration of 1,5% NaCl at 15°C and 37°C; the role of NaCl concentration was tested on cells grown in TSB with final concentration of 3% NaCl and TSB with final concentration of NaCl of 5%; finally the iron concentration influence was investigated on cells grown in TSB plus 10 µM of EDDA to create an iron limitation condition and TSB plus 5 µl, 10 µl and 20 µl of Fe^{3+}.

3.2.3 Bacterial growth and samples collection.

Bacterial grown was monitored using a biophotometer (Eppendorf BioPhotometer). At $O.D._{600}$ values of 0.1, 0.6, 0.9, corresponding to lag exponential and early stationary phase, aliquots of the broth culture were collected for CFU count and for real time PCR analyses. Aliquots of 100 µl of pure culture 10-fold serially diluted were spread in sterile Tryptic soy agar (TSA) (oxioid) and incubated at 25° for 48 h. Only plates showing CFU between 30 and 300 colonies were counted.

3.2.4 DNA and RNA extraction from bacterial cells.

DNA and RNA were extracted from 2 ml of *Vibrio anguillarum* pure culture at the chosen $O.D._{600}$ using Qiagen RNA/DNA Mini Kit (Qiagen).

The cells were harvested by centrifugation at 3000-5000 x g at 4 °C and, each of sample, was treated with lysozime and QRL1 solution and placed into the column.

The extraction was carried out as according to the manufacture's instructions. DNA and RNA were stored in isopropanol at –20°C before the precipitation. The DNA and RNA were resuspended in 50 µl of RNase free water.

The DNA was used as template for further molecular analysis.

RNA extracted was treated with "TURBO DNA-free" kit (Ambion) to eliminate residual of DNA in the final elution.

cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen). Mixture (final volume 20µl) for each reaction contained 4 µl of 5X First-Strand Buffer, 3µl of template RNA, 1 µl (10 µM) of Random Primers, dNTP (10mM each) and 1 µl of SuperScript II RT (200 units).

The cDNA synthesis was at 42°C for 50 min. The reaction was stopped by heating at 70°C for 15 min.

The cDNA was used as the template for a further Real Time PCR amplification.

The quality and concentration of DNA and RNA were determined using the NanoDrop® ND-1000 Spectrophotometer (Celbio).

3.2.5 Primers and specificity.

The primer sets specific for the target genes of *V. anguillarum* was newly designed in this study using Primer Express software (Primer Express software, version 2.0 Applied Biosystems, Foster City, Calif.) with reference to the partial sequence of *V. anguillarum*

published in GeneBank (Table 1). The sequences of the primer sets used for real time PCR analysis are shown in Table 1. The sequences of fragments obtained by PCR analysis using as template cDNA from *V. anguillarum* strains, were checked by BLAST research in the GenBank database (**http://www.ncbi.nml.nih.gov**).

Table 1: Primers used in this study. *Melting temperature is calculated by Primer Express software version 2.0.

Gene/ Primer	Sequence Forward (5'-3')	Sequence Reverse (5'-3')	Amplicon length (bp)	Melting Temperature (°C)*	Source of reference	GeneBank number
empA	CAATTTGCTGCGCCATCAT	CGTAGCAACGCAAACTGAGAAA	81	77	In this study	LO2528
rpoN	GTGCTCGTATTGCAGTCGATGA	AATTCGAAAATACCACGCGG	91	78	In this study	AB214574
toxR	ACACCACCAACGAGCCTGA	TTGTCTCTTCGGGTTGCGA	90	80	In this study	AB042543
tonb2	TTTTACAGCGCGTTGGTTACC	GCCAATTGCAGGAGCGATT	82	80	In this study	AM259386
OMP	CAGACAACAATGCAGATGGCT	GATACAGCAGCGAATGCGTC	90	82	In this study	FJ573227
fur	TTCACCCGGCTTTTTTATTGTC	TGTGCCGACGGCAGTTG	85	81	In this study	L33344
angR	GGGACGCTATTCCACATGGT	CCGGCGGCACTATCGTAT	90	80	In this study	M34504
fatA	ACTCTTTTGGTGGCGAGCAA	ACGCTGACTGGTGGTATCGC	80	79	In this study	AY312585

3.2.6 Real-time PCR and cycling parameters.

Real Time PCR was run in a 7300 Real Time PCR System (Applied Biosystems, Foster City, Calif.) in triplicate. Reaction mixture was prepared using SYBR® Green PCR Master Mix Applied Biosystems in a total volume of 25 µl containing 12,5 µl of Master Mix, 0,5 µl of primers forward and reverse (at optimized concentrations), 1 µl of cDNA containing 70ng from pure culture sample. Sterile MilliQ water was used to adjust the volume of each reaction to 25 µl.

A no-template control (NTC) and a positive control DNA from pure culture were included on each plate.

The thermal cycling protocol included an initial denaturation step at 95 °C for 10 min,

followed by 45 cycles of denaturation at 95°C for 15 s, annealing/elongation at 60°C for 60 s. Dissociation step was added to check for primer-dimer formation.

3.2.7 Data analysis using the $2^{-\Delta\Delta Ct}$ method.

Data evaluation was performed using the ABI Prism 7300 Sequence Detection System. Differential expression of target genes under stress condition of growth was determined by the relative quantitative comparative threshold cycle method ($\Delta\Delta Ct$) using *rpoN* as housekeeping gene and standard condition of growth as calibrator (Thiel *et al*. 2003; ABI PRISM User Bulletin # 2). The $\Delta\Delta Ct$ method normalizes the detected fluorescent signal to an endogenous reference gene and subsequently compares target signals in different samples to a calibrator sample; the amount of gene target is given by $2^{-\Delta\Delta Ct}$. For all experiments, each individual sample was run in triplicate wells and the Ct of each well was recorded at the end of the reaction.

3.2.8 Design of standard curve for validation test.

A ten-fold dilution series, ranging from 10 to 10^5 ng, of cDNA extracted from pure culture of *Vibrio anguiallarum* per reaction, was used to create a standard curve for each gene for the validation test. The concentration of cDNA was measured using NanoDrop® ND-1000 Spectrophotometer (Celbio).

Standard dilutions were analyzed in triple. The Ct values were plotted against the logarithm of their initial template concentration. Standard curves were generated by a linear regression of the plotted points.

3.3. Results

3.3.1 Comparison of growth curves of Vibrio anguillarum grown under stress condition.

Vibrio anguillarum strains were grown in batch cultures to determine the effect of

temperature , salinity and iron availability on growth rate and viability (table 2 -3).

Table 2. Characteristics of *Vibrio anguillarum* 975\I cells batch grown in TSB under several environmental stress conditions.

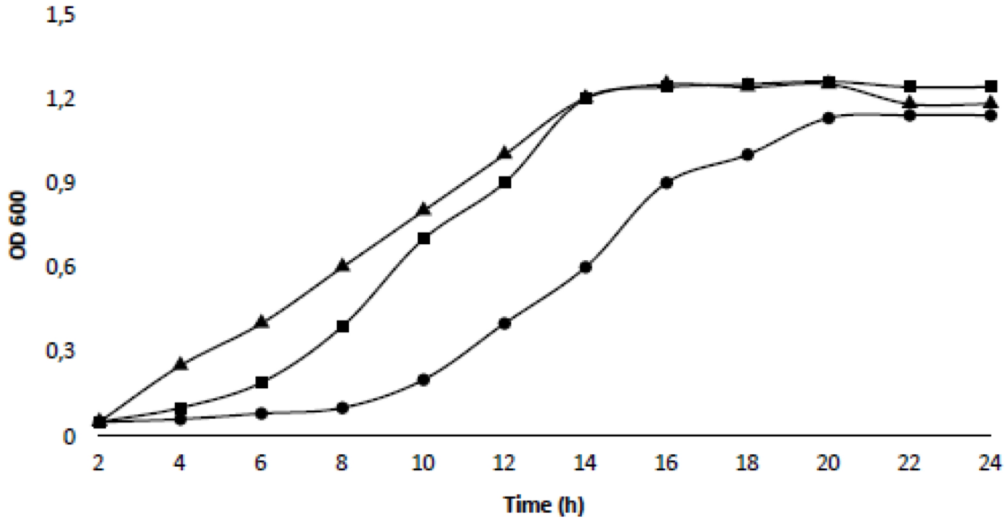
Media	Growth conditions		Final density (CFU\ml)	Growth rate (h^{-1})
TSB + 1,5% NaCl	T (°C)	15 °C	$1,2 \times 10^{10}$	0,16
		25 °C	$5,6 \times 10^{10}$	0,20
		37 °C	$5,4 \times 10^{10}$	0,25
TSB 25 °C	NaCl concentration (%)	1,5 %	$5,6 \times 10^{10}$	0,20
		3 %	$4,3 \times 10^{10}$	0,22
		5 %	$1,2 \times 10^{10}$	0,16
TSB+1,5%NaCl 25°C	Iron concnetration (µM)	EDDA 10µM	7×10^{9}	0,17
		Fe^{3+} 5µM	$3,2 \times 10^{10}$	0,20
		Fe^{3+} 10µM	$2,3 \times 10^{10}$	0,23
		Fe^{3+} 20µM	$1,4 \times 10^{10}$	0,19

Table 3. Characteristics of *Vibrio anguillarum* ATCC 43307 cells batch grown in TSB under several environmental stress conditions.

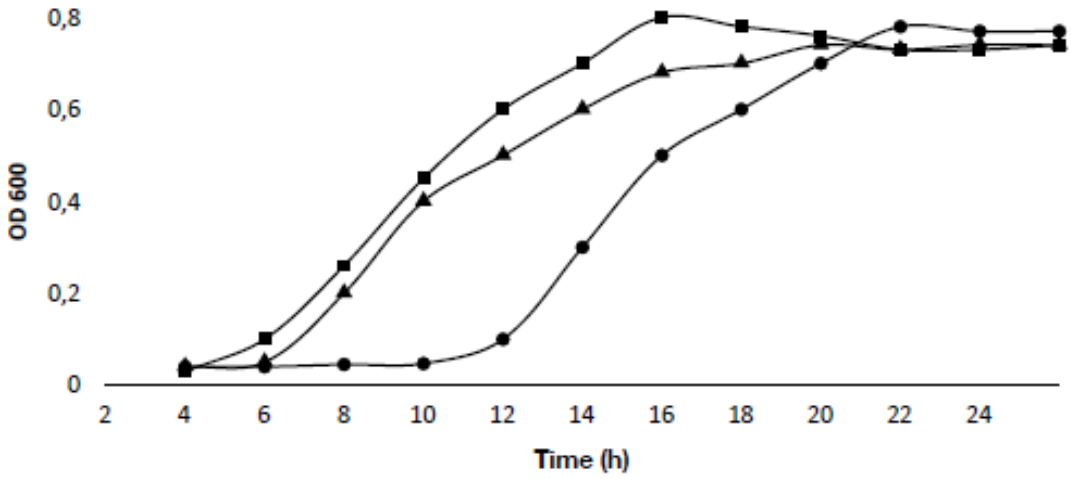
Media	Growth conditions		Final density (CFU\ml)	Growth rate (h^{-1})
TSB + 1,5% NaCl	T (°C)	15 °C	$1,2 \times 10^{9}$	0,10
		25 °C	$3,4 \times 10^{9}$	0,12
		37 °C	$2,3 \times 10^{9}$	0,11
TSB 25 °C	NaCl concentration (%)	1,5 %	$3,4 \times 10^{9}$	0,10
		3 %	$1,8 \times 10^{9}$	0,11
		5 %	$5,6 \times 10^{8}$	0,15
TSB+1,5%NaCl 25°C	Iron concnetration (µM)	EDDA 10µM	$5,9 \times 10^{8}$	0,08
		Fe^{3+} 5µM	$1,3 \times 10^{9}$	0,13
		Fe^{3+} 10µM	$1,5 \times 10^{9}$	0,14
		Fe^{3+} 20µM	$1,7 \times 10^{9}$	0,13

Figure 1 a and b show the growth curves of *Vibrio anguillarum* 975/I and *Vibrio anguillarum* ATCC43307 grown in TSB with final concentration of 1,5% of NaCl cultured at different temperatures. The results showed that at 15°C;there were the longer lag pahses corresponding to 10 h in *Vibrio anguillarum* 975/I and 12h in *Vibrio anguillarum* ATCC 43307. The temperature range comprised between 25°C and 37°C seems to not influence the growth performance of the two strains. In *Vibrio anguillarum* 975/I at 25°C and 37°C the maximum turbidity was reached after 14h (OD_{600} 1.2); in comparison at 15°C to reach the maximum OD_{600} 1,1, 22h were required (figure 1 a).

In *Vibrio anguillarum* ATCC43307 was appreciable a positive effect at 37°C; in this condition of growth the maximum turbidity was reached in 14h (OD_{600} 0.8) while at 15°C that maximum OD_{600} of 0,8 was reached in 22h (figure 1 b).



a.



b.

Figure 1 a-b. Growth cures of *Vibrio anguillarum* 975/I serovar O1 (a) and *Vibrio anguillarum* ATCC 43307 serovar O3 (b) grown in TSB with final concentration of NaCl of 1,5% at 25°C, consider the optimal condition of growth (square) and in TSB + 1,5% of NaCl at 15°(circle) and at 37°C (triangle).

Figure 2 a and b show the growth curves of *Vibrio anguillarum* 975/I and of *Vibrio anguillatum* ATCC 43307 grown in TSB cultured in different concentrations of NaCl and incubated at 25°C.

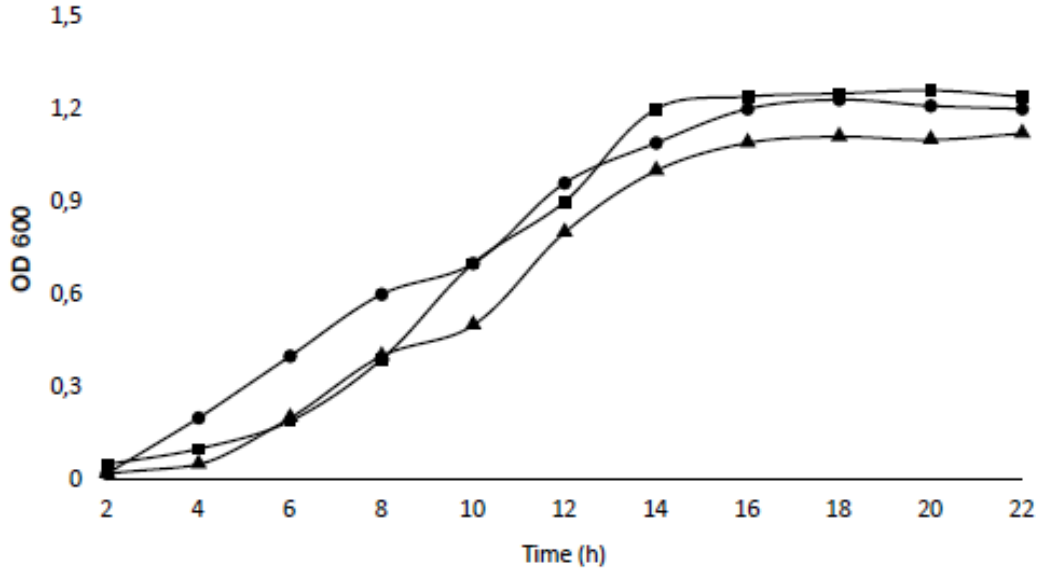
Vibrio anguillarum 975/I grown equally in TSB supplemented with NaCl at different concentrations of 1,5%, 3% and 5%; for all condiction tested the maximum OD_{600} of

1,2 was reached after 18h incubation.

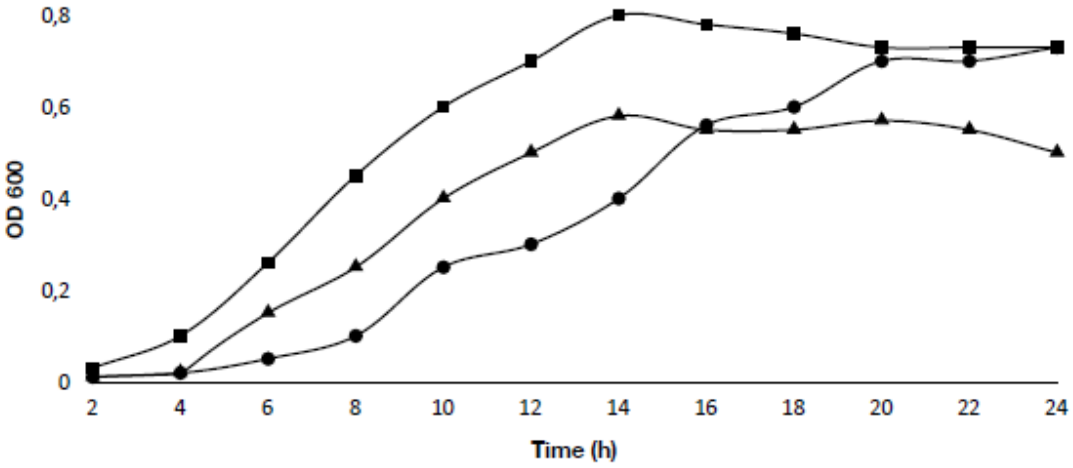
Figure 2 b shows the growth curves of the plasmidless strain, ATCC43307. The results showed different growth curves during the osmotic stress; at a concentration of 3% of NaCl the lag phase was the longest (6 h) while at the concentration of NaCl of 5% was recorded a lag phase of 4 h. Moreover, the maximum OD_{600} of 0,7 was reached in 22h during the incubation in TSB with final concentration of 3% of NaCl.

Figure 3 a and b show the growth curves of *Vibrio anguillarum* 975/I and *Vibrio anguillarum* ATCC 43307, respectively, grown in TSB with final concentration of 1,5% of NaCl cultured in different concentrations Fe^{3+} (5 μM, 10 μM and 20 μM) and cultured in iron starvation condition by the addiction of EDDA (50 μM). Incubations were carried out at 25°C.

The trends of the curves showed differences between the conditions tested for both strains; during the iron starvation condition were recorded the longest lag phases corresponding to 6 h and 8 h in *Vibrio anguillarum* 975/I and ATCC 43307, respectively. When the iron in the growth media was added until to reach the concentration respectively of 5 μM, 10μM and 20μM of Fe^{3+}, the trend of the growth didn't show appreciable differences. In *Vibrio anguillarum* 975/I (figure 3 a) the maximum OD_{600} of 1,2 was reached in 14h while in *Vibrio anguillarum* ATCC43307 the maximum OD_{600} of 0.8 was reached in 10h.

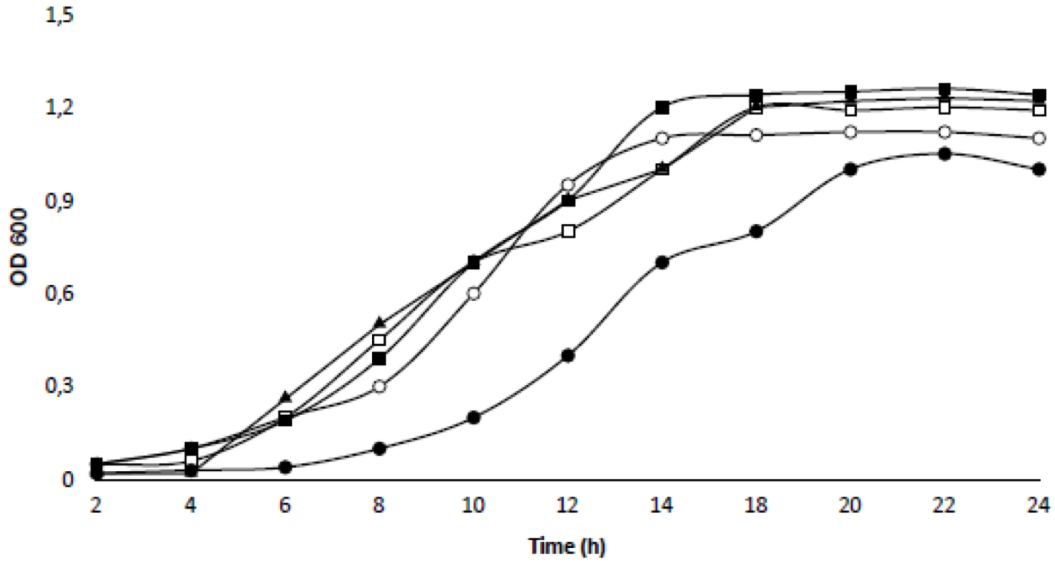


a.

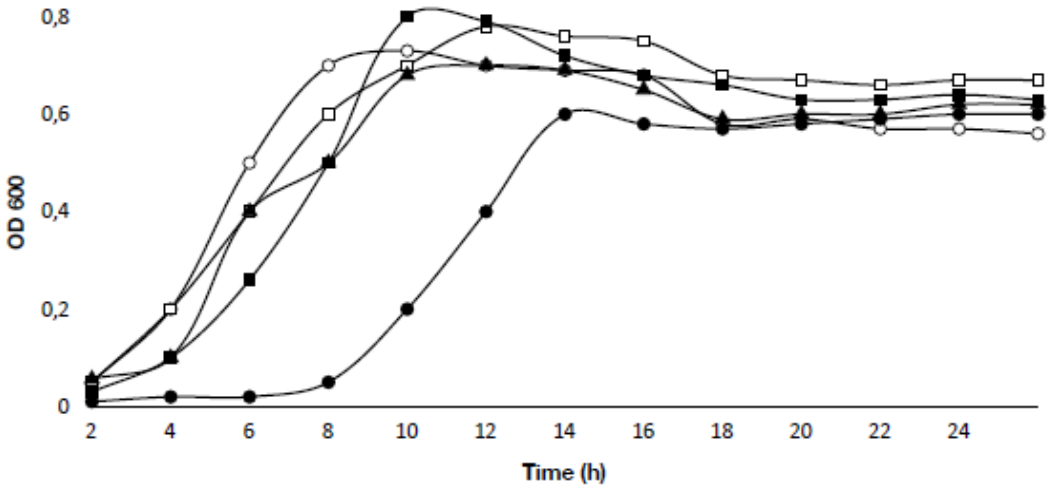


b.

Figure 2 a-b. Growth curves of *Vibrio anguillarum* 975/I serovar O1 (a) and *Vibrio anguillarum* ATCC 43307 serovar O3 (b) grown in TSB with final concentration of NaCl of 1,5% (square), 3% (triangle) and 5% (circle) at 25°C.



a.



b.

Figure 3 a-b. Growth curves of *Vibrio anguillarum* 975/I serovar O1 (a) and *Vibrio anguillarum* ATCC 43307 serovar O3 (b) grown in TSB with final concentration of NaCl of 1,5% at 25°C (square); in TSB + 1,5% of NaCl with the addiction of 50 µM of EDDA (circle); in TSB + 1,5% of NaCl with the addiction of 5µM of Fe3+(triangle), 10 µM of Fe3+ (open square) and 20 µM of Fe3+ (open circle).

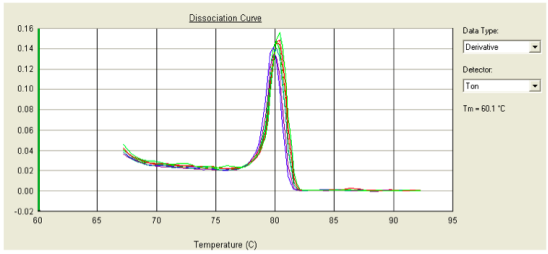
3.3.2 Primers specificity

DNA and cDNA from pure cultures of *V.anguillarum* strains were successfully PCR-amplified with all tested primers (table 1), with the exception of the primers designed on plasmid genes, *fatA* and *angR* genes; DNA and cDNA from pure culture of *Vibr*io *anguillarum* ATCC 43307 didn't show PCR amplified with *angR* and *fatA* primers (data not shown).

In the figures 4 (a-b-c-d-e-f-g-h) are showed the specificity of the corresponding amplification products observed during a melting curve analysis between 60°C and 90°C, only specific amplification showed a single peak at the expected temperature, namely 78°C for rpoN primers; 80°C for toxR primers; 82°C for OMP primers; 80°C fro tonB primers; 81°C for furR primers; 77°C for empA primers; 80°C for angR primers and 79°C for fatA primers.

The identities of the PCR amplicons from the isolates were confirmed by sequencing. The sequence data showed that *V. anguillarum* strains contained 100% identical nucleotides over the fragment investigated.

a.



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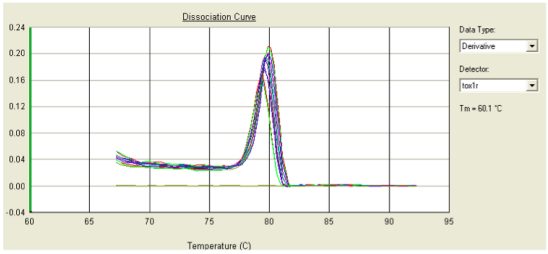
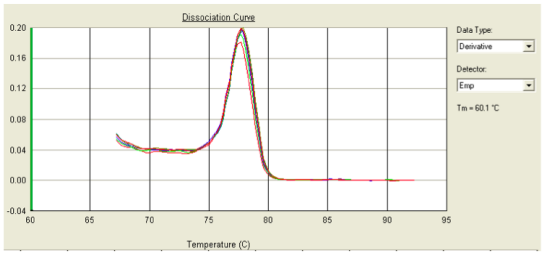
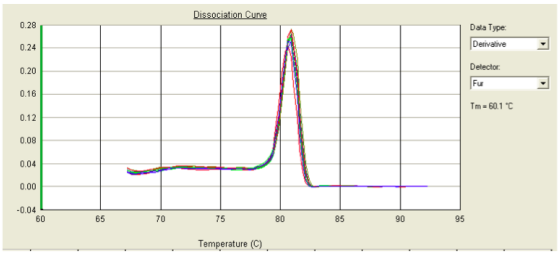


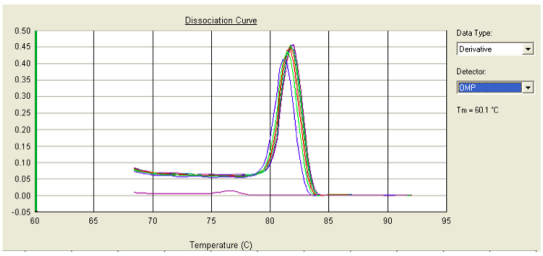
c.



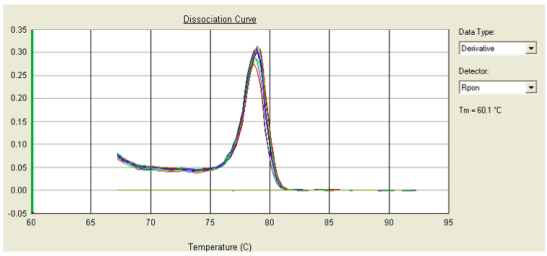
d.



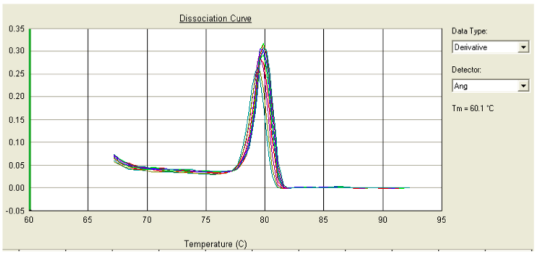
e.



f.



g.



h.

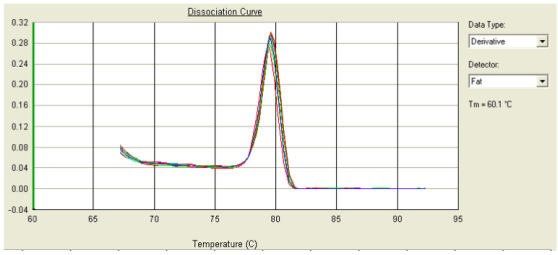


Figure 4 a-b-c-d-e-f-g-h-i. Dissociation curves analysis using tonB2 primers (melting temperature 80°C) (a) ; toxR primers (melting temperature 80°C) (b); empA primers (melting temperature 82°C)(c); Fur primers (melting temperature 81°C) (d); OMP primers (melting temperature 82°C)(e); rpoN primers (melting temperature 78°C)(f); angR primers (melting temperature 80°C)(g); fatA primers (melting temperature 79°C)(h).

3.3.3 Validation test.

To verify the validity of the method and the quantitative measurement, cDNA purified from a pure culture of *V. anguillarum* sampled at $O.D._{600}$ 0,6 was serial-diluted (10-fold dilutions) and analyzed by real time PCR.

Figure 5 shows standard curves of tested genes.

The values of each slope, indicated in the figure 5, fall within the optimal range (between -3.3 and -3.4 ABI PRISM User Bulletin # 2), the Ct values of each target gene tested and the Ct of the housekeeping gene were maintained constant. The cDNA amount to use as template for further analyses was within the range where slopes were linear.

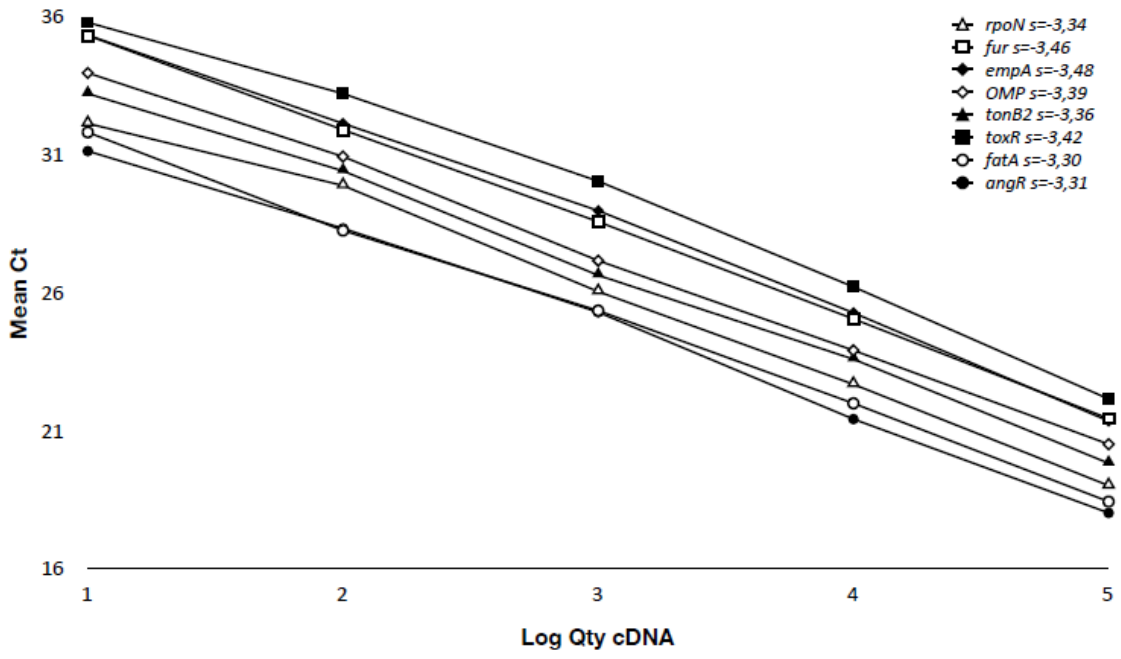


Figure 5. Validation test plotted from triplicate samples using Ct values of tenfold dilutions of cDNA template extracted from *V. anguillarum* 975\I. The curves confirmed the comparability of *tonB2, toxR, empA, Fur, OMP, angR, fatA* target genes respect *rpoN* housekeeping gene, the slope values are maintained between -3,3 and -3,4 and the Ct values for each gene respect the housekeeping are constant.

3.3.4 Differential expression of virulence genes under temperature stress.

To examine the influence of temperature on the expression of target genes in *Vibrio*

anguillarum, 975/I and ATCC43307, the strains were incubated in TSB+1,5% of NaCl at temperatures of 15°C, 25°C and 37°C. The total RNA was extracted from cells during the exponential phase of growth.

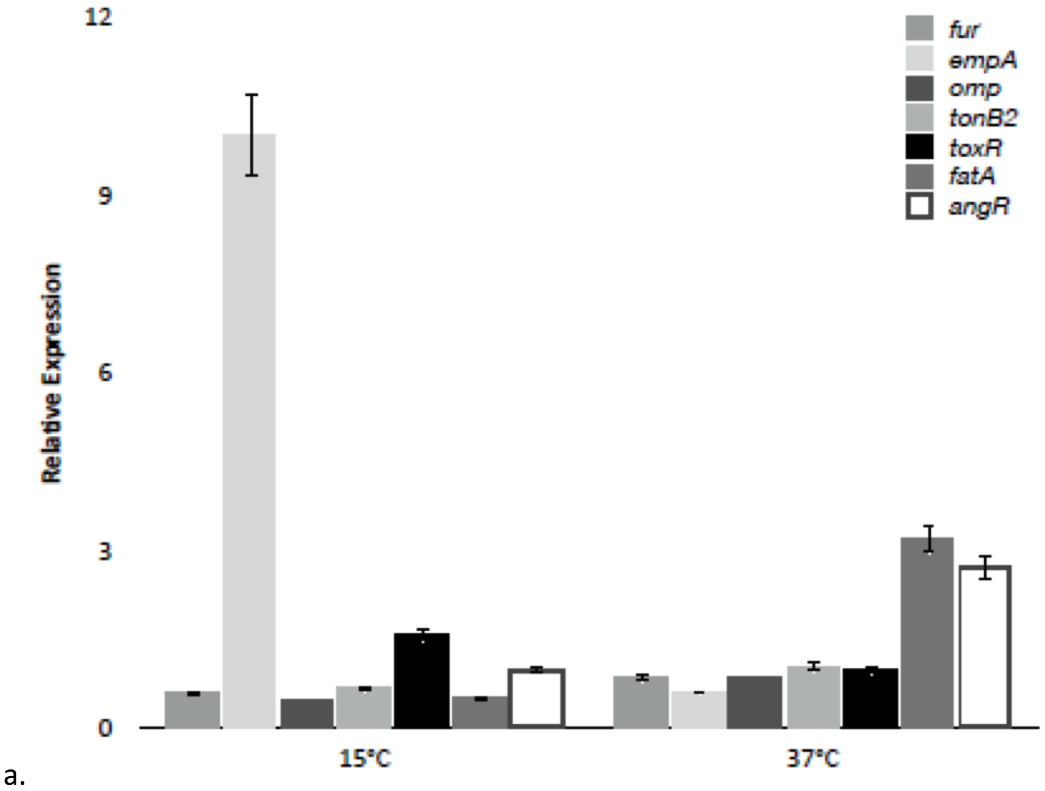
The relative expression levels of target genes under experimental temperatures, compared to the optimal condition of growth are collected in the figure 6 (a-b).

In the strain 975/I, that carries the plasmid pJM1, the results (figure 6 a) showed that, at 15°C the *empA* gene was strongly over expressed; the calculation of $2^{-\Delta\Delta Ct}$ has showed that *empA* expression was 10 fold more than 25°C. During the incubation at 37°C the *empA* expression decreases until to be under-expressed while the differential expression of *angR* and *fatA* genes was, approximately, 4 time more compared to expression at 25°C.

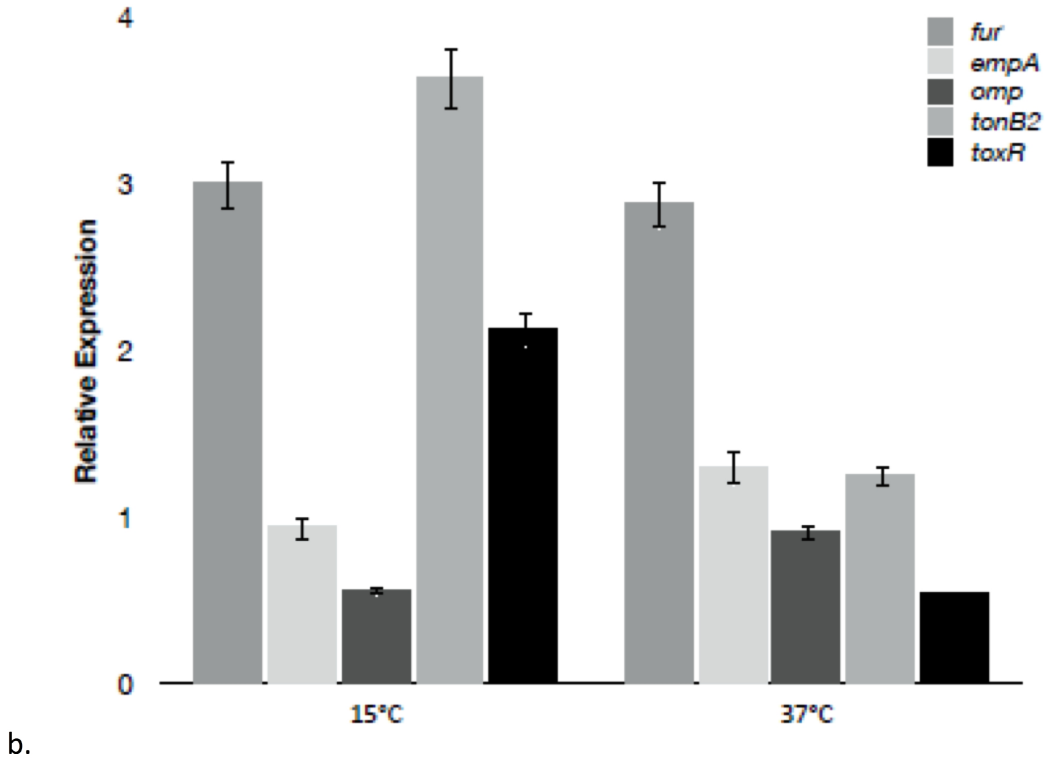
The variations of the virulence genes expression under temperature stress in *Vibrio anguillarum* 03, plasmidless strain, were significantly different (figure 7 b).

During the incubation at 15°C was estimated the over expression of *fur* and *tonB2* genes, the expression for both genes was 3,5 times more than 25°C; moreover the expression of *toxR* gene was 2 fold the values registered at 25°C.

At 37°C was stable the over-expression of *furR*, 3 fold the value registered at 25°C, while the expression of *toxR* and *tonB2* decreases (figure 7 b).



a.



b.

Figure 6 a-b. Differential expression of target genes during the incubation of cells at 15°C and 37°C compared to the standard condition of growth (25°C) in *Vibrio anguillarum* 975/I (a) and *Vibrio anguillarum* ATCC 43307 (b), y-axis shows the $2^{-\Delta\Delta Ct}$ of target genes at the different temperatures compared to basal expression (1).

3.3.5 Differential expression of virulence genes under osmotic stress.

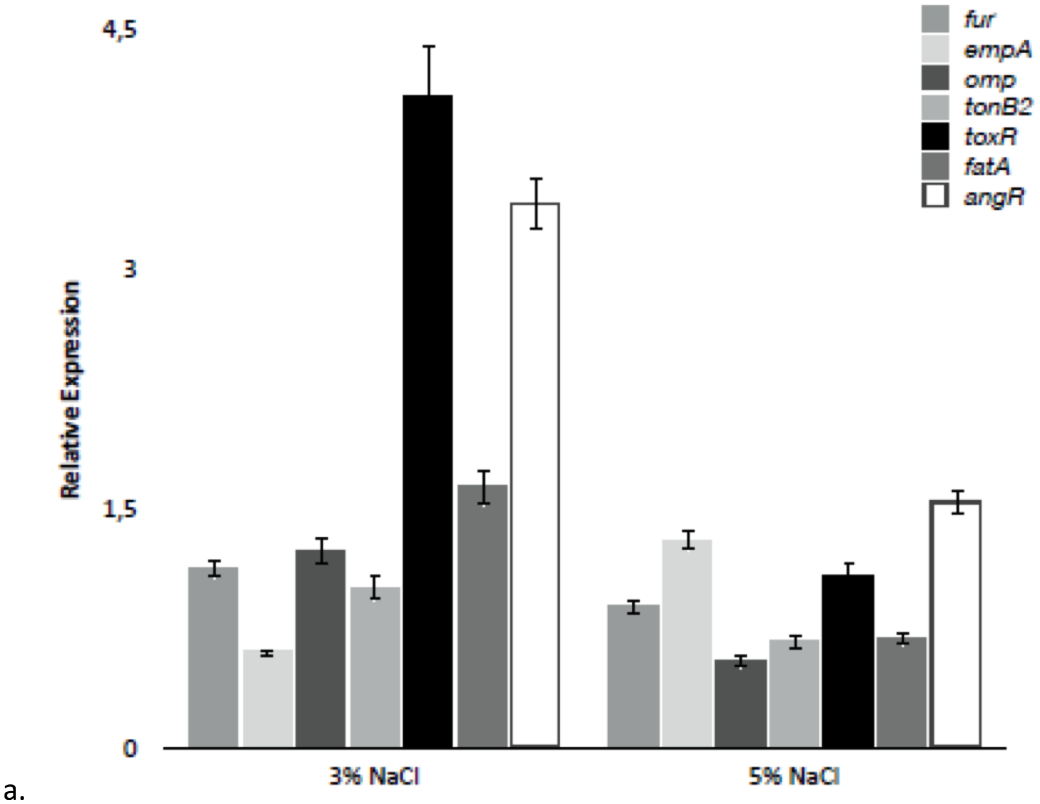
The differential expression of target genes was tested during the exposition of *Vibrio anguillarum* strains at different concentration of NaCl in the growth media.

The total RNA analyzed was extracted from *Vibrio anguillarum* 975/I and *Vib*rio *anguillarum* ATCC43307 during the exponential phase of growth. The relative expression levels of target genes compared to the optimal condition of growth are collected in the figure 7 (a-b).

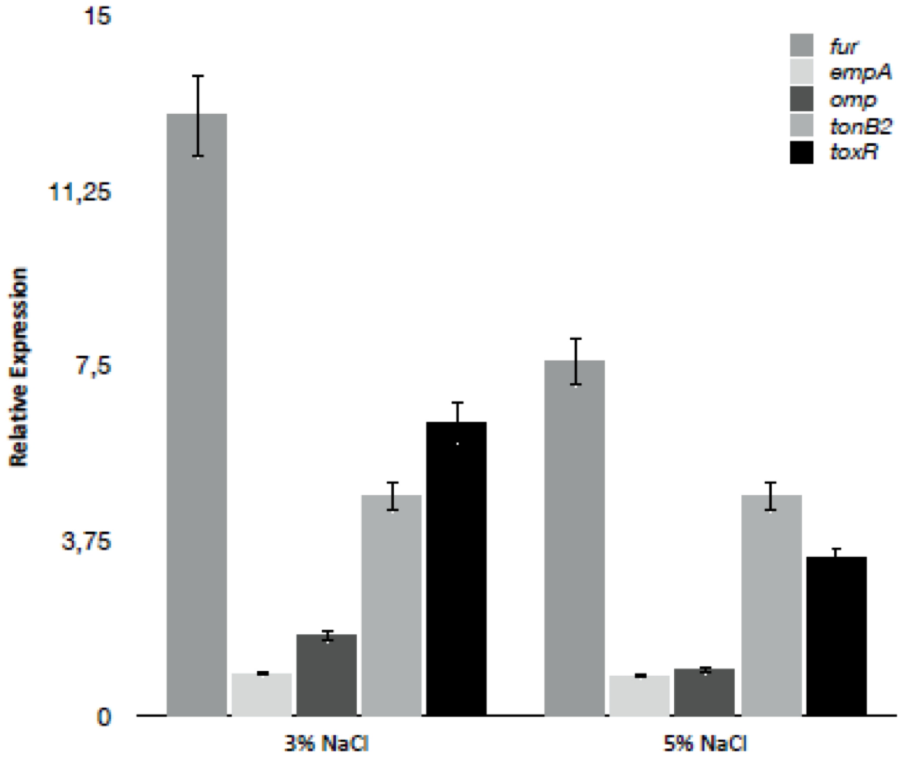
The results of differential expression of target genes for the strain 975/I, are collected in the figure 7 a. The histograms showed that, at the concentration of 3% of NaCl, was recorded the over expression of *toxR* and *angR* gene, the $2^{-\Delta\Delta Ct}$ values were 4 and 3,5 times more, respectively, compared to the optimal condition of growth. When sodium chloride reached the final concentration of 5% the expression of cited genes decreases.

Concerning the strain ATCC43307 (figure 7 b), at the concentration of 3% of NaCl *fur* gene was 12 times more expressed as well as at the concentration of 5% of NaCl the expression was 8 fold the value registered at the optimal condition of growth.

Moreover, for both concentrations of NaCl tested, the expression of *OMP* was approximately 4 times more compared to expression of *OMP* at the optimal condition of growth wile the *toxR* expression at the concentration of 3 % and 5 % of NaCl was, respectively, 6,2 and 3,27, time more than the optimal condition of growth.



a.



b.

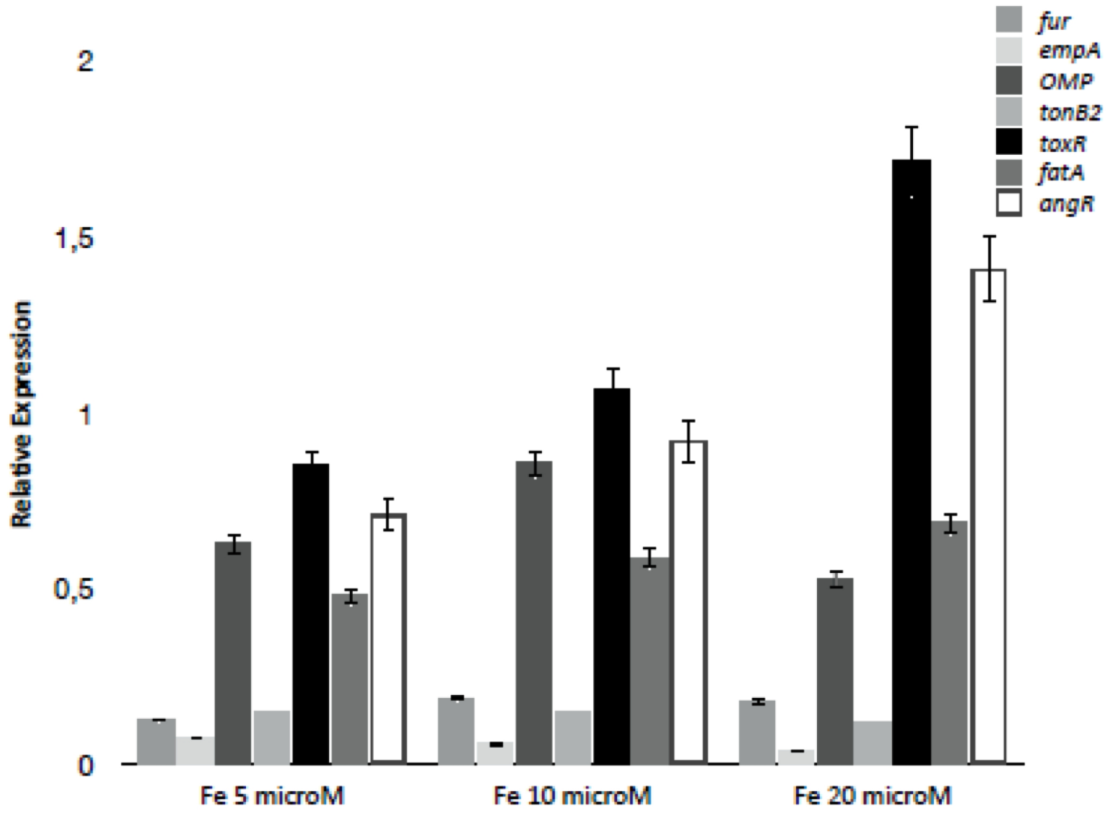
Figure 7 a-b. Differential expression of target genes during the incubation in 3% and 5% of NaCl in *Vibrio anguillarum* 975/I (a) and *Vibrio anguillarum* ATCC 43307 (b) compared with the optimal condition of growth (1,5% of NaCl); y-axis shows the $2^{-\Delta\Delta Ct}$ of target genes at the different temperatures compared to basal expression (1).

3.3.6 Differential expression of virulence genes under iron stress.

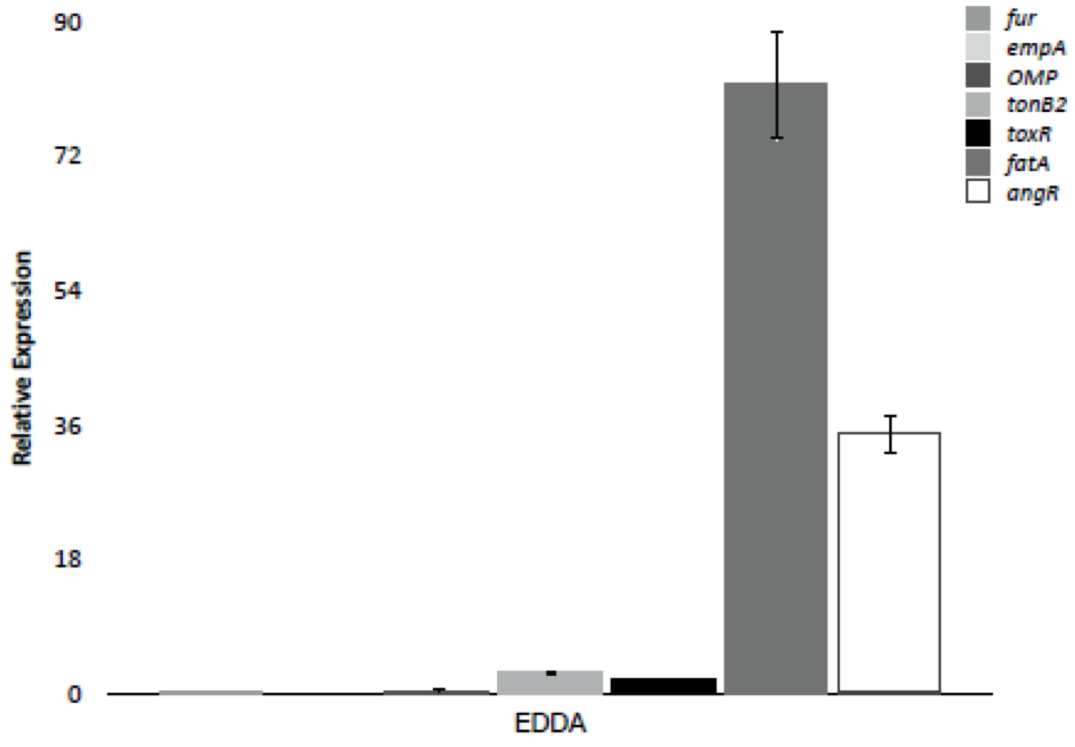
To examine the influence of iron availability on the expression of target genes in *Vibrio anguilarum*, 975/I and ATCC43307, the strains were incubated in TSB+1,5% of NaCl with the addiction of EDDA, to create iron starvation condition, and in TSB + 1,5% of NaCl with the addiction of 5, 10 and 20 μM of Fe^{3+}. The total RNA was extracted from cells during the exponential phase of growth.

The relative expression levels of target genes under experimental stress, compared to the optimal condition of growth, are collected in the figure 8 (a-b) and in the figure 9.

In the serovar O1 the increase of iron in the growth media didn't determine any differential expression of target genes comparing it to the expression of the same genes during an optimal condition of growth (baseline of expression is 1). Only when the iron reaches a concentration of 20 μM was recorded a moderate response of *toxR* and *angR* genes, the $2^{-\Delta\Delta Ct}$ values were, respectively, 1,7 and 1,2. During the incubation in iron starvation condition the plasmidic genes, *angR* and *fatA* showed a high over expression; *fatA* gene was 81,57 times more expressed while *angR* was 34,93 time more expressed compared with the optimal condition of growth (figure 8 b).



a.



b.

Figure 8 a-b. Differential expression of target genes (b) during a condition of iron depletion, with the addiction of EDDA 50µM, (a) and during serial addition of Fe^{3+} (5µM, 10µM and 20µM)(b) in *Vibrio anguillarum* cells 975/I compared with the optimum condition of growth; y-axis shows the $2^{-\Delta\Delta Ct}$ of target genes at the different temperatures compared to basal expression (1).

In the strain ATCC43307 (figure 9) the addition of iron stimulated the over-expression of *toxR* that at the concentration of iron of 10 µM showed an expression 5 times more compared to the expression of *toxR* at the optimal condition of growth; the trend of expression of *toxR* gene was maintained constant also during the incubation of cells in iron limiting condition. Moreover, in iron limiting condition, was recorded the over expression of *tonB2*; the expression of cited gene was 12,86 time more than the optimal condition of growth.

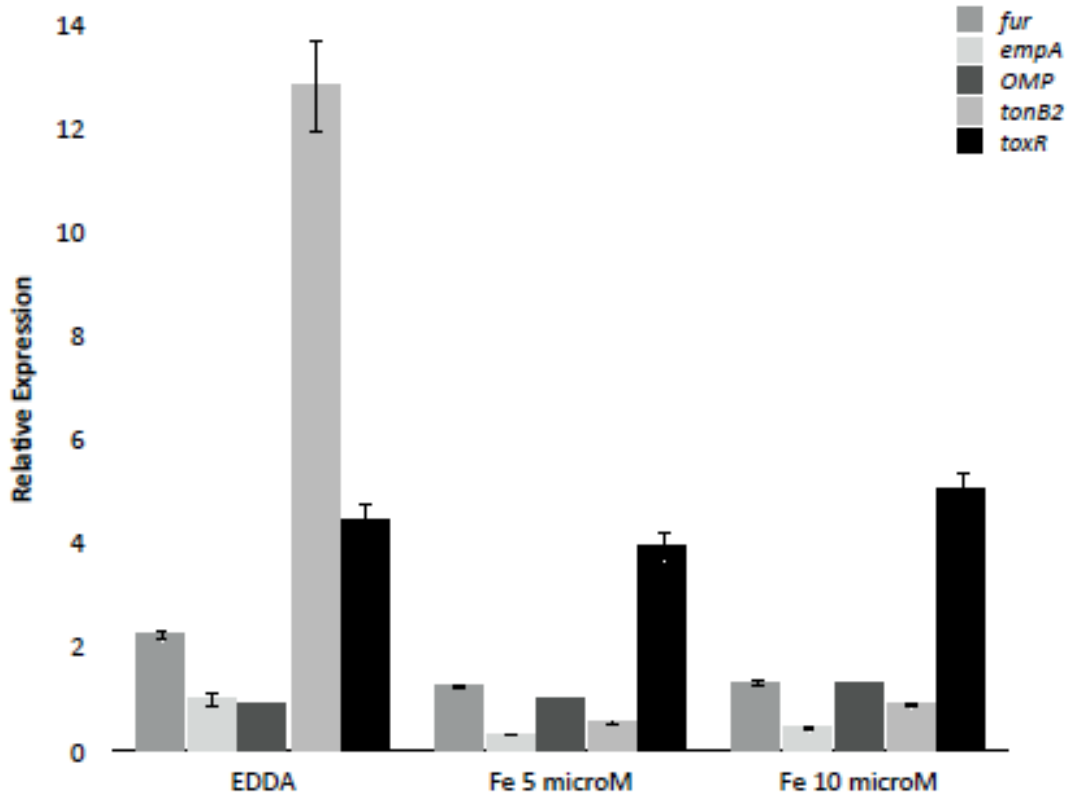


Figure 9. Differential expression of target genes during a condition of iron depletion, with the addiction of EDDA 50µM, and during serial addition of Fe^{3+} (5µM, 10µM and 20µM) in *Vibrio anguillarum* ATCC 43307 cells compared with the optimum condition of growth; y-axis shows the $2^{-\Delta\Delta Ct}$ of target genes at the different temperatures compared to basal expression (1).

3.4 Discussion

Several authors have demonstrated that environmental stress influences particular virulence factors in *Vibrio anguillarum*, such as mobility (Larsen et al. 2004) and production of extracellular substances (Weber et al. 2008), but little is known about the expression of virulence genes.

In this study we present results demonstrating that the expression of virulence genes in *Vibrio anguillarum* strains, belonging to serovar O1 and serovar O3, was coordinately regulated by several environmental factors and that there is a specific response expression of target genes to variations in several conditions of growth.

The first evidence of our results was that, for all growth conditions tested, the strain plasmid-bearing (*Vibrio anguillarum 975/I* serovar O1) showed a more enhanced growing performance than the plasmidless strain (*V*. *anguillarum* ATCC43307 serovar O3), this suggest that the tolerance to environmental variations was better in the virulent strain in comparison with an avirulent strain.

The analysis on the expression profile of virulence genes by Real Time PCR, demonstrated that the gene expression is specifically induced and by variations in environmental parameters.

Iron depletion was the environmental condition producing the most dramatic change in *Vibrio anguillarum* 975/I: the expression of *angR* and fatA genes was 80 and 40 folds higher, respectively, than optimal conditions of growth.

Our results agree with the model proposed by Stork *et al.* (2002) (Fig 10), the anguibactin iron-uptake system is quickly induced under condition of iron starvation;the response of anguibactin system is detectable after 10h from the exposition to the modified medium.

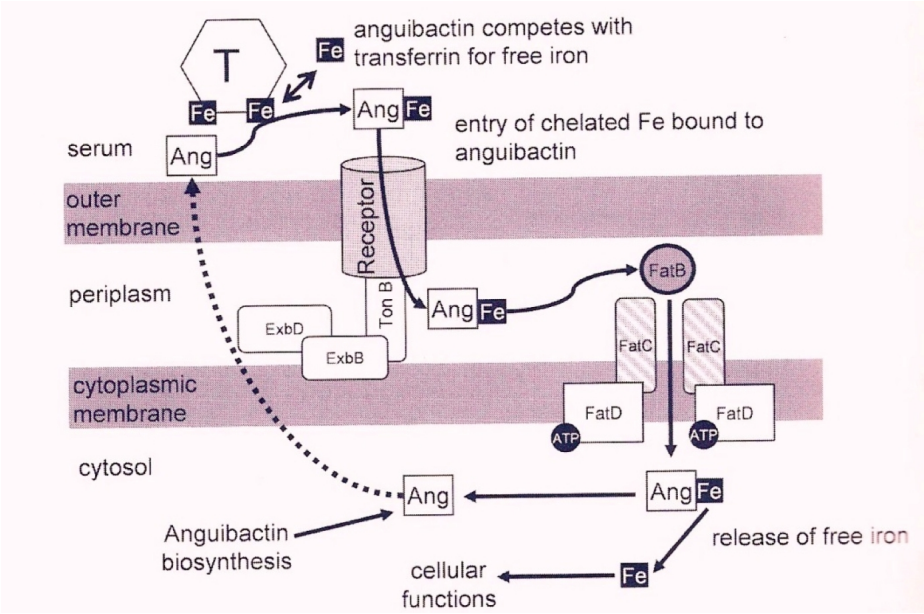


Figure 10. Schematic of the anguibactin mediated iron uptake system.

In comparison during the iron depletion exposure expression of *tonB2* was reached 12 fold in the plasmidless strain. *tonB2* is involved in the transport of iron inside the cell, its increased expression suggests the possible existence of an alternative iron-uptake system for the strains which lack pJM1-like plasmids as suggested by Lemos *et al.* 2010.

Temperature can be considered, on the basis of our results, a parameter that potentially influences the pathogenicity in *Vibrio anguillarum* serovar O1; at 15°C expression of the empA gene was 10-fold higher than that at optimal temperature of growth. This result is described in detail in the next chapter.

Our results suggest that the effect of environmental stress on virulence factors is very different in the strains studied; in *Vibrio anguillarum* 975/I serovar O1 carrying pJM1 plasmid, the genes strongly induced by environmental parameters were *empA*, *angR* and *fatA,* which have an important role during the infection process in fish, while in

Vibrio anguillarum ATCC43307 serovar O3 the genes that showed a highest influence were *toxR* an *fur* which regulate phenotypic modification in relation to environmental changes in several pathogens (Hahan *et al*. 2009; Martinez-Picado *et al.* 1996; Thompson *et al*. 2004; Zhu *et al.* 2002; Elias *et al.* 2009; Wung *et al*. 1998; Bidle *et al.* 2001; Abdallah *et al.* 2009).

The capacity to resist to sudden phisico-chemical variation in the environment is a peculiar feature of pathogens specially conferred by the presence of plasmid (resistance to the antibiotic, metals, pollution, etc.).

Curing experiments are in progress to verify the resistance to environmental stress of *Vibrio anguillarum* 975/I deprived of pJM1 plasmid.

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Chapter 4

"Influence of temperature, salinity (NaCl%) and growth media on the production of EmpA metallo-protease in *Vibrio anguillarum*."

4. Influence of temperature, salinity (NaCl%) and growth media on the production of EmpA metallo-protease in Vibrio anguillarum.

4.1 Introduction

V. anguillarum protease is an elastolytic metalloprotease dependent on Zn^{2+} for its activity and Ca^{2+} for its stability. According to Milton *et al* (1992) EmpA is synthesized as a 66.7-kDa preproenzyme. Sequence analysis predicts that the removal of both pre- and propeptides during secretion would result in a mature protein with a molecular mass of 44.6-kDa. However, EmpA protease activity was repeatedly associated with a 36-kDa protein (Milton *et al.* 1992); this suggests that the 44.6-kDa protein undergoes further processing to a 36-kDa active form. Staroscik *et al.* (2005) used Western blot analysis to study EmpA secretion in *V. anguillarum* culture supernatants; using anti-LasB antibodies for detection, Western blot analysis revealed a 46-kDa band in all culture supernatants as well as a 36-kDa band that could be detected only in culture supernatants possessing protease activity (Staroscik *et al.* 2005). The 46- and 36-kDa bands correspond to the sizes of the predicted secreted proenzyme and the mature protein, respectively (Milton *et al*. 1996). Staroscik *et al.* (2005) also confirmed the presence of the cytoplasmic preproenzyme. Moreover, according to Varina et al exist a single gene of *V. anguillarum* (epp) was identified and characterized with regard to its ability to promote the processing of extracellular pro-EmpA to mature EmpA. (Varina *et al.* 2008).

As other proteases produced by pathogens, EmpA contributes to infection in at least two ways: first, as a part of the protein quality control machinery required for the turnover of unfolded proteins generated in the adverse host environment. Secondly,

growing evidence supports a conserved role in specific and controlled proteolysis of regulatory proteins in response to temporal, spatial or environmental stimuli (Ingmer and Brondsted 2009).

In this context, Weber *et al.* (2008) have reported in an *in vitro* study a cascade pathway control on the production of protease EmpA by *Vibrio anguillarum* switched on and driven by quorum sensing (high cell density). Moreover Denkin and Nelson (1999 and 2004) have demonstrated the inducer effect of gastrointestinal mucus of salmon added to the growth medium on EmpA production in *Vibrio anguillarum*. The same work also shows that the importance of the composition of growth medium. In fact, many authors have shown that the nature of nutrients and their relative concentration also influence the production of proteases.

Other environmental parameters, such as temperature and salinity, have been identified as possible inducers of proteases production. For example in *Burkholderia pseudomallei* extracellular proteases have been determined in the secretome following a salt stress with increased levels ranged from 9.97 to 143.85 fold, the maximal degree detected in the study (Pumirat *et al.* 2010). Moreover, Khan *et al.* (2007) reported that the protease production in the human pathogen *Aeromonas sobria* (able to tolerate various environmental conditions) is repressed when the pathogen is in sea water respect the normal production in river water (Khan *et al.* 2008), this phenomenon can be explained as an adaptive behavior to marine environment.

The aim of this work is the study of the influence variation of temperature, concentration of NaCl and growth media, on the expression of *empA* gene and the effective production, under the tested conditions in *Vibrio anguillarum 975\I*.

4.2. Materials and Methods

4.2.1 Bacterial strains and Media.

The bacterial strain used in this study was *Vibrio anguillarum* serotype O1 975\I a local (Mediterranean Sea) virulent strain (kindly provided by Dr. Manfrin, Istituto Zooprofilattico delle Venezie – Italy) isolated from sea bass specimens.

Cells were routinely grown in Tryptic Soy Broth (Pancreatic Digest of Casein 17.0 g\l, Enzymatic Digest of Soybean Meal 3.0 g\l, Sodium Chloride 5.0 g\l, Dipotassium Phosphate 2.5 g\l, Dextrose 2.5 g\l supplemented with 1% of NaCl) and on TCBS plates (Thiosulfate-Citrate-Bile Salts-Sucrose supplemented with 0.5% of NaCl) at 25°C.

To test the influence of growth conditions on protease production, *Vibrio anguillarum* cells were grown in different experimental media.

Experimental media included cM9 (6 g\l Na_2HPO_4, 3 g\l KH_2PO_4, 5 g\l NaCl 1 g\l NH_4Cl 0,01M $CaCl_2$ 0,1M $MgSO_4$ ph 7.2), a minimal culture medium supplemented with Casamino acids (0,2%) and Glucose (0,5%) and ONR7a (22g\l NaCl, 3,98g\l Na_2SO_4 1,3g\l TAPSO, 0,72 g\l KCl, 0,27 g\l NH_4Cl, 0,089 g\l Na_2HPO_4x7H2O, 0,083g\l NaBr, 2,6 mg\l NaF, 31mg\l $NaHCO_3$, 27mg\l H_3BO_3, 11,18g\l $MgCl_2$ 6H2O, 1,46g\l $CaCl_2xH_2O$, 24mg\l $SrCl_2$ x $6H_2O$, 2 mg\l $FeCl_2$ x $4H_2O$) supplemented with Casamino acids (0,2%) and Glucose (0,5%), an artificial seawater mineral salts medium based on the ionic composition of seawater, this medium contained all of the major cations and anions that are present at concentrations greater than 1 mg/liter in seawater. Incubations were carried out under moderate agitation (150rpm).

To test the response to temperature stress, the cultures were incubated at 15°C and 37°C using the media above described.

4.2.2 Bacterial growth and samples collection.

Bacterial growth was monitored using a biophotometer (Eppendorf BioPhotometer). At $O.D._{600nm}$ values of 0.1, 0.6, 0.9, corresponding to lag, exponential and early stationary phase, aliquots of the broth culture were collected for CFU count and for real time PCR analyses. Aliquots of 100 µl of pure culture 10-fold serially diluted were spread in sterile Tryptic soy agar (TSA) (oxioid) and incubated at 25°C for 48 h. Only plates showing CFU between 30 and 300 colonies were counted.

4.2.3 DNA and RNA extraction from bacterial cells.

DNA and RNA were extracted from 2 ml of *Vibrio anguillarum* pure culture at $O.D._{600nm}$ using Qiagen RNA/DNA Mini Kit (Qiagen).

The cells were harvested by centrifugation at 3000-5000 x g at 4 °C and, each of sample, was treated with lysozime and QRL1 solution and placed into the column.

The extraction was carried out as according to the manufacture's instructions. DNA and RNA were stored in isopropanol at –20ºC before the precipitation. The DNA and RNA were resuspended in 50 µl of RNase free water.

The DNA was used as template for further molecular analysis.

RNA extracted was treated with "TURBO DNA-free" kit (Ambion) to eliminate residual DNA in the final elution.

cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen). Mixture (final volume 20µl) for each reaction contained 4 µl of 5X First-Strand Buffer, 3µl of template RNA, 1 µl (10 µM) of Random Primers, dNTP (10mM each) and 1 µl of SuperScript II RT (200 units).

The cDNA synthesis was at 42°C for 50 min. The reaction was stopped by heating at 70°C for 15 min.

The cDNA was used as the template for a further Real Time PCR amplification.

The quality and concentration of DNA and RNA were determined using the NanoDrop® ND-1000 Spectrophotometer (Celbio).

4.2.4 Primers and specificity.

The primer sets specific for the *empA*, *vanT, rpoS and rpoN* genes of *V. anguillarum* were newly designed in this study using Primer Express software (Primer Express software, version 2.0 (Applied Biosystems, Foster City, Calif.) with reference to the partial sequence of *V. anguillarum* published in GeneBank (respectively LO2528; AF457643; AY695433; U86585). The sequences of the primer sets used for real time PCR analysis are shown in Table 1.

The sequences of fragments were checked by BLAST research in the GenBank database (**http://www.ncbi.nml.nih.gov**)

Table 1: Primers used in this study.* Melting temperature is calculated by Primer Express software version 2.0.

Gene/ Primer	Sequence Forward (5'-3')	Sequence Reverse (5'-3')	Amplicon length (bp)	Melting Temperature (°C)*	Source of reference
empA	CAATTTGCTGCGCCATCAT	TCGTTCCCGGGTTAAGTTAATTT	81	77	In this study
rpoN	GTGCTCGTATTGCAGTCGATGA	AATTCGAAAATACCACGCGG	91	78	In this study
rpoS	AGCACCGGCTAACTCCGTG	TGCGCTTTACGCCCAGTAAT	90	76	In this study
vanT	CCTGGTAGTCCACGCCGTAA	CCAGGCGGTCTACTTAACGC	92	80	In this study

4.2.5 Real-time PCR and cycling parameters.

Real Time PCR was run in a 7300 Real Time PCR System (Applied Biosystems, Foster City, Calif.) in triplicate. Reaction mixture was prepared using SYBR® Green PCR Master Mix Applied Biosystems in a total volume of 25 µl containing 12,5 µl of Master Mix, 0,5 µl of primers forward and reverse (at optimized concentrations), 1 µl of cDNA containing 70ng from pure culture sample. Sterile MilliQ water was used to adjust the volume of each reaction to 25 µl.

A no-template control (NTC) and a positive control DNA from pure culture were included on each plate.

The thermal cycling protocol included an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing/elongation at 60°C for 60 s. Dissociation step was added to check for primer-dimer formation.

version 2.0.

4.2.6 Data analysis using the $2^{-\Delta\Delta Ct}$ methods and its validation.

Data evaluation was performed using the ABI Prism 7300 Sequence Detection System. Differential expression of target genes under stress condition of growth was determined by the relative quantitative comparative threshold cycle method ($\Delta\Delta Ct$) using *rpoN* as housekeeping gene and standard condition of growth as calibrator (Thiel *et al*. 2003; ABI PRISM User Bulletin # 2). The $\Delta\Delta Ct$ method normalizes the detected fluorescent signal to the endogenous reference gene and subsequently compares target signals in different samples to the calibrator sample. The amount of gene target is given by $2^{-\Delta\Delta Ct}$. For all experiments, each individual sample was run in triplicate wells and the Ct of each well was recorded at the end of the reaction.

4.2.7 Design of standard curve for validation test.

A ten-fold dilution series, ranging from 10 to 10^5 ng, of cDNA extracted from pure culture of Vibrio anguiallarum per reaction, was used to create a standard curve for each gene for the validation test. The concentration of cDNA was measured using NanoDrop® ND-1000 Spectrophotometer (Celbio).

Standard dilutions were analyzed in triple. The Ct values were plotted against the logarithm of their initial template concentration. Standard curves were generated by a linear regression of the plotted points. The slope of each curve was measured fir the

validation test.

4.2.8 Protease activity.

V. anguillarum cells were grown overnight in TSB, cM9, and ONR7a to the stationary phase.

Culture supernatants were assayed for proteolytic activity by using a modification of the method described by Windle and Kelleher. Culture supernatant was incubated with azocasein (5 mg/ml) dissolved in Tris-HCl (50 mM [pH 8.0]) containing 0.04% NaN_3.

Culture supernatant was prepared by centrifuging 1 ml of cells (12,000 x g, 10 min). Supernatant was removed and filtered through a 0.22-µm -pore-size cellulose-acetate filter. Filtered supernatant (100 µl) was incubated at 37°C with 100 µl of azocasein solution for 1 h. The azo-casein reaction time was determined by performing assays on V. anguillarum supernatants from all the experimental media and incubation temperature. Reactions were terminated by addition of trichloroacetic acid (10% [wt/vol]) to a final concentration of 6.7% (wt/vol). The mixture was allowed to stand for 1 to 2 min and centrifuged (12,000 × g, 4 min) to remove unreacted azocasein, and supernatant containing azopeptides was suspended in 700 µl of 525 mM NaOH.

In order to determine the rate of protease activity induction by culture media, *Vibrio anguillarum* cells were grown overnight at 25°C in TSB + 1,5% of NaCl; after this incubation the cells were centrifuged (90000 X g, 10 min) and pelleted cells were washed twice with NSS. Washed cells were resuspended to the appropriate cell density in experimental media including TSB, cM9 and supernatant of *Vibrio anguillarum* pure culture grown in cM9 overnight at 25°C. In order to determine the induction of experimental condition on protease activity, every hour for 4 hours, the

samples was collected and then treated for the azo-casein assay (as described above).

4.2.9 Preparation of proteins extracts.

Supernatant was collected from *V. anguillarum* cultures grown overnight in either TSB + 5%of NaCl, cM9 or ONR7a (both supplemented with o,2% of Casamino acids and 0,5% of glucose). Aliquots (1 ml) of the cultures were centrifuged (9,000 x g, 10 min, 4°C) to pellet cells, and supernatants were filtered through 0.22-µm-pore-size filters (Millipore, Billerica, Mass.). Protein was precipitated with 10% tricholoroacetic acid (4°C overnight), pelleted by centrifugation (12,000 X g, 15 min, 4°C), rinsed twice with acetone, and resuspended in 15 µl of 1X phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 x $7H_2O$, and 1.5 mM KH_2PO_4). Supernatant proteins were used for western blot analysis.

4.2.10 Western Blot.

An aliquot of 1ml supernatant proteins were precipitated with 10% TCA (AppliChem) for 1h in ice, pelleted by centrifugation to 20,000 x g for 30 min at 4°C, rinsed twice with acetone (Sigma-Aldrich) and resuspended in 2X Laemmli sample buffer (100mM Tris·HCl pH 6.8, 4% SDS, 20% glycerol, 0.2% Bromophenol Blue, 80mM DTT). The samples were denatured for 10 min to 100°C and separated on a 10% SDS-PAGE using, as standard molecular weight, Prestained Protein Molecular Weight Marker (Fermentas, Life Sciences). Gel was transferred to 0.45 µm nitrocellulose membrane (Hybond ECL, Amersham BioSciences) using Mini-Protean II system (BioRad) at 100V for 1h. The membranes were blocked with 10% skim milk (BioRad) and incubated with rabbit anti-EmpA 1:10,000 (kindly provided by Debra Milton) for 1h at 37°C. After washing with TBS-Tween buffer, the membranes were incubated 1h at room temperature with HRP conjugated goat anti-rabbit. Specific signals were detected

with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer).

4.3 Results.

4.3.1 Influence of culture media on the growth curves.

Vibrio anguillarum strain 975/I was grown in batch cultures to determine what effect medium, salinity and temperature have on growth rate and viability (table 2).

Table 2. Characteristics of V*ibrio anguillarum* 975\I cells batch grown in TSB+ 1,5% NaCl, cM9, ONR7a at 15°C, 25°C and 37°C.

Medium	Temperature	Finally Density (CFU\ml)	Growth Rate (h^{-1})
TSB + 1,5% NaCl	15 °C	$3,2 \times 10^{10}$	0,16
	25 °C	$1,4 \times 10^{10}$	0,27
	37 °C	$7,8 \times 10^{11}$	0,31
cM9	15 °C	$2,5 \times 10^{8}$	0,11
	25 °C	$1,8 \times 10^{9}$	0,15
	37 °C	$6,2 \times 10^{8}$	0,18
ONR7a	15 °C	$1,5 \times 10^{8}$	0,08
	25 °C	$3,4 \times 10^{9}$	0,13
	37 °C	$6,2 \times 10^{9}$	0,14

Fig. 1 a shows the growth curves of *Vibrio anguillarum* 975/I in TSB with final concentration of NaCl of 1,5% cultured at different temperatures. The results showed a different trend of the growth during the incubation at 25°C, 37°C and 15°C; in the last condition was recorded the longer lag phase corresponding to 12 h. The temperature range comprised between 25°C and 37°C seems to not influence the

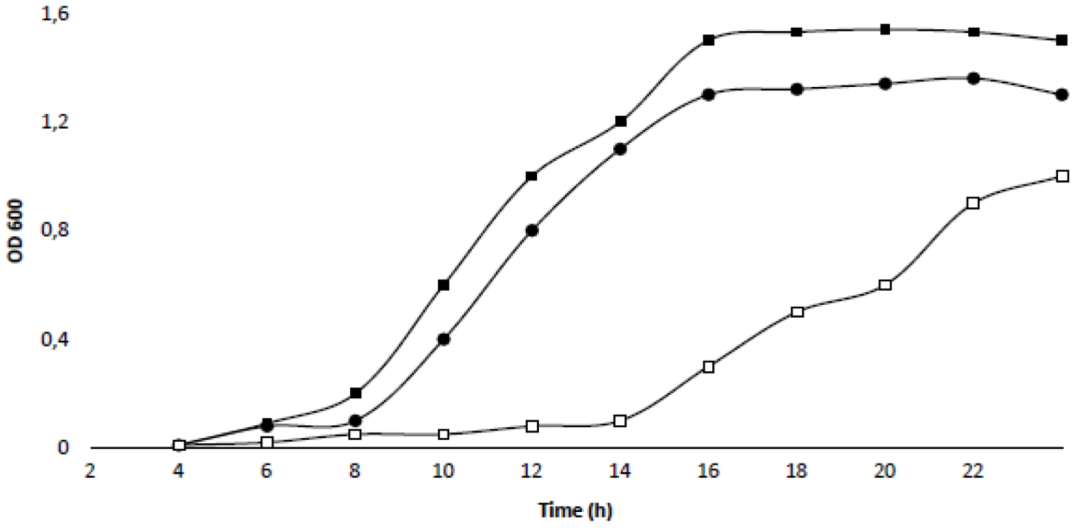
growth performance of the strain, except for a positive effect at 37°C reaching the maximum turbidity in 14h (OD_{600} 1.4). On the contrary at 15°C to reach the maximum OD_{600} 0.9, 22h were required.

Fig. 1 b shows the growth curves of *Vibrio anguillarum* inoculated in cM9. In this case the trends of curves is different for the three temperatures tested: at 15°C the exponential phase started after a lag phase of 10 h reaching the maximum turbidity after 24h with a cell density of 10^8; in addiction at 37°C was recorded the highest growing performance between all temperatures tested, with a maximum OD_{600} reached of 1,4.

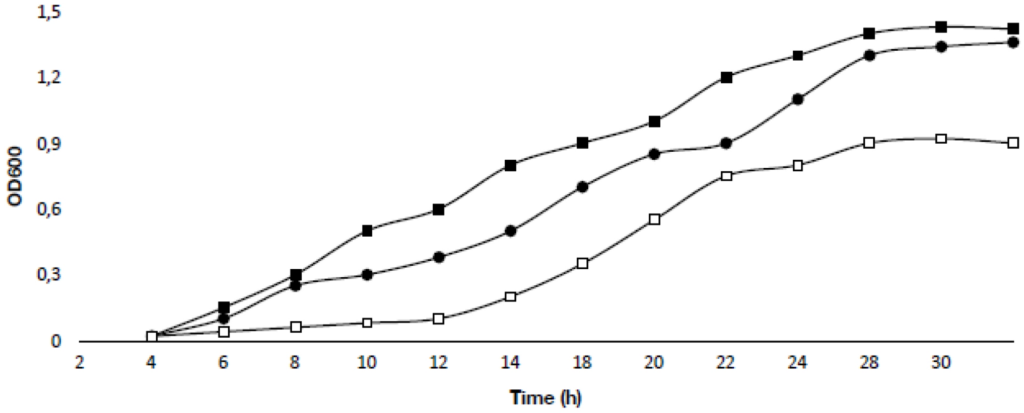
Fig. 1 c shows the growth curves of *Vibrio anguillarum* 975/I grown in ONR7a.

The curves showed a clear influence of the temperature on the *Vibrio anguillarum*. Also in this case, the more stressful condition, analyzing the lag phase, is 15°C corresponding to 10 h. At 25°C and 37°C were recorded the same lag time, while the maximum OD_{600} reached were different corresponding to 0,8 at 25°C and 1 at 37°C.

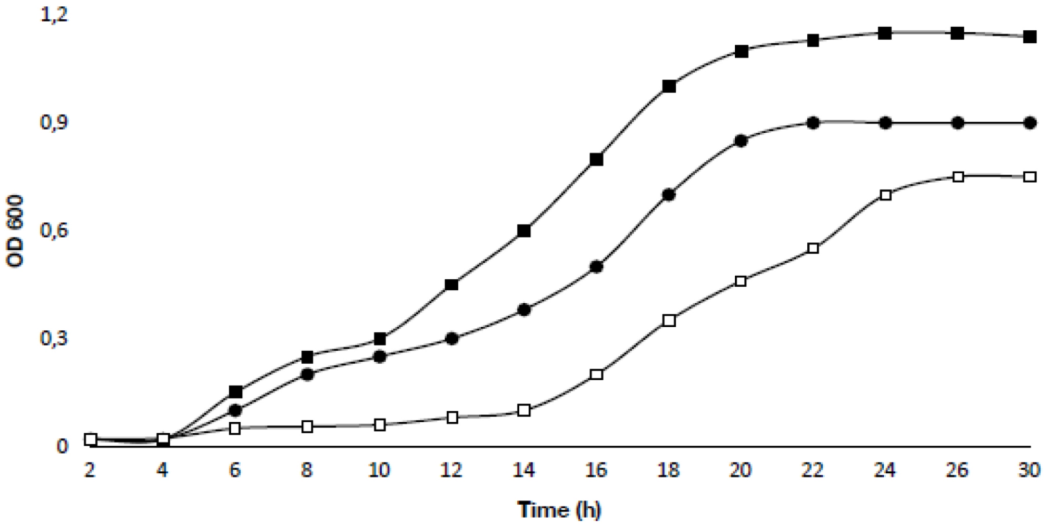
As indicated in table 2 the minimum growth rate was registered at 15°C in all tested medium, while a cumulative effect of temperature and salinity was visible on ONR7a, where *Vibrio anguillarum* shows the lowest growth performance.



a.



b.



c.

Figure 1 a-b-c. Growth curves of *Vibrio anguillarum* incubated in TSB+1,5%NaCl (a); in cM9 supplemented with Casamino acids (0,2%) and glucose (0,5%) (b) and ONR7a supplemented with casamino acids (0,2%) and glucose (0,5%) (c) at 15°C(open square), 25°C (circle) and 37°C (square). X-axis indicates the time in hours and y-axis the OD_{600} values.

4.3.2 Primers design and specificity.

cDNA from pure cultures of *Vibrio anguillarum* was successfully PCR-amplified with all tested primers (table 1) Figure 2 a-b-c-d shows the specificity of the corresponding amplification products observed during a melting curve analysis between 60°C and 95°C, only specific amplification showed a single peak at the expected temperature, namely 77°C for empA primers; 78°C for rpoN primers; 76°C for rpoS primers and 80°C for vanT primers.

The identities of the PCR amplicons from the isolates were confirmed by sequencing.

The sequence data showed that *V. anguillarum* strains contained 100% identical nucleotides over the fragments investigated.

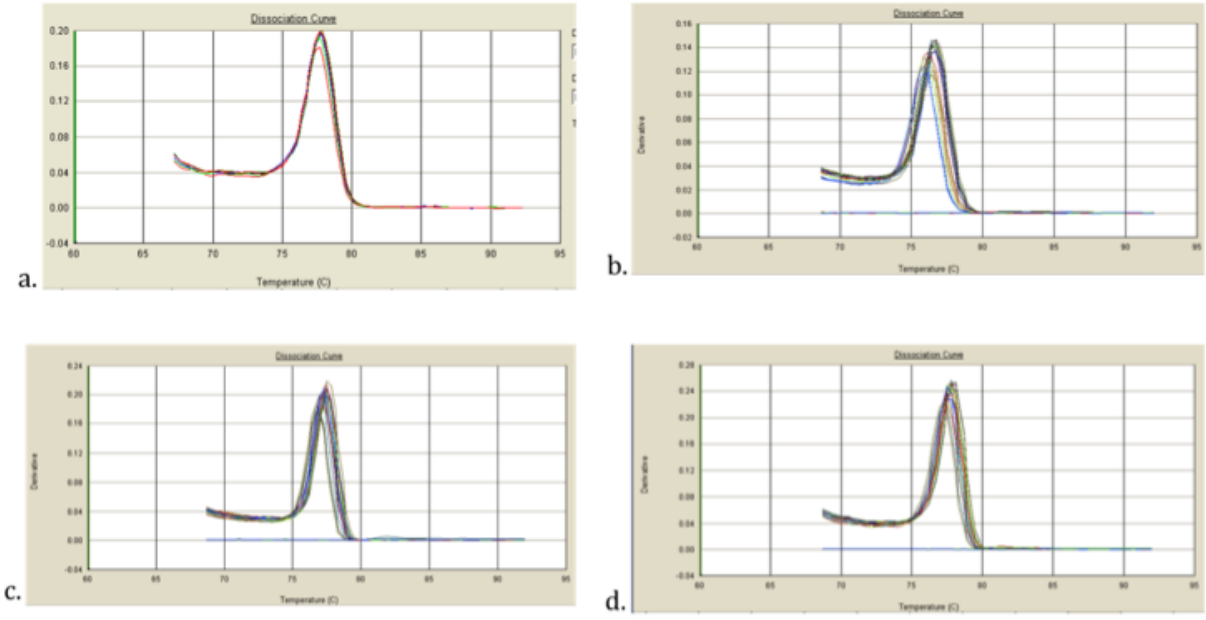


Figure 2 a-b-c-d. Dissociation curves analysis using empA primers (melting temperature 77°C) (a) ; rpoS primers (melting temperature 76°C) (b); rpoN primers (melting temperature 78°C) (c) and vanT primers (melting temperature 80°C) (d).

4.3.3 Validation test.

To verify the validity of the method and the quantitative measurement, cDNA purified from a pure culture of *V. anguillarum* sampled at O.D. $_{600}$ 0,6 was serial-diluted (10-fold dilutions) and analyzed in real time PCR.

Figure 3 shows the standard curves of *rpoS, empA, vanT, rpoN* genes. From the average of Ct values SyBR Green assay is exponential over abroad dynamic range, from 10 to at least 10^5 CFU starting material.

The slopes values, namely, -3.34 for *rpoN*, -3.48 for *empA,* -3.32 for *vanT,* -3.36 for *rpoS,* fall within the optimal range (between -3.3 and -3.4 ABI PRISM User Bulletin # 2), the Ct values of each target gene tested and the Ct of the housekeeping gene were maintained constant. The cDNA amount to use as a template for further analyses was within the range were slopes were linear.

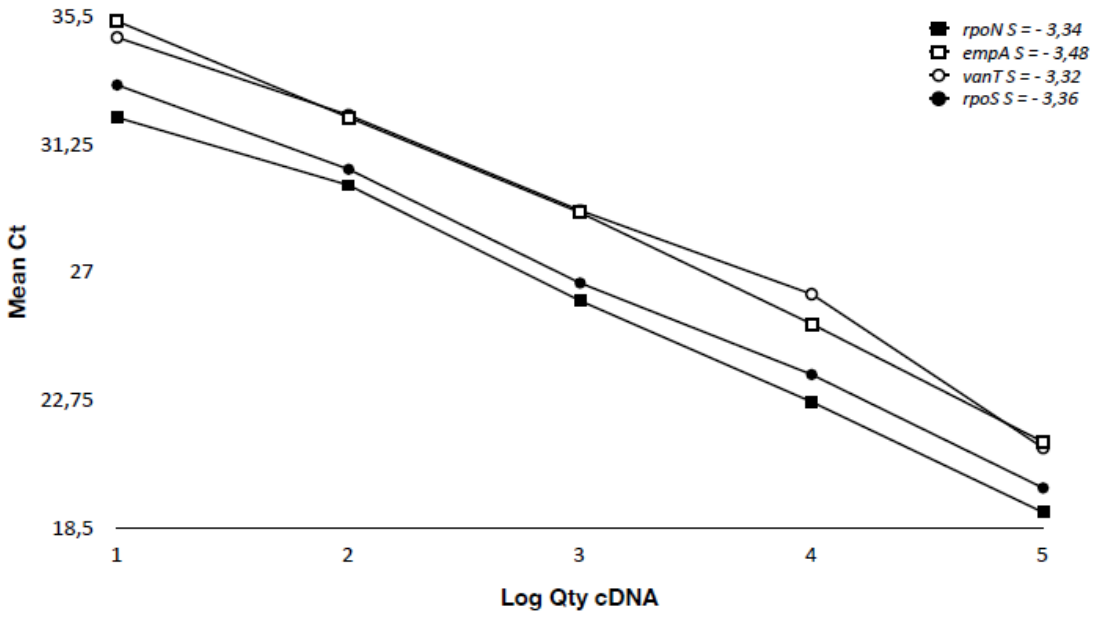


Figure 3. Validation test plotted from triplicate samples using Ct values of tenfold dilutions of cDNA template extracted from *V. anguillarum* 975\I. The curves confirmed the comparability of *emp, vanT, rpoS* target genes respect *rpoN* housekeeping gene, the slope values are maintained between -3,3 and -3,4 and the Ct values for each gene respect the housekeeping are constant.

4.3.4 Influence of temperature on empA expression in Vibrio anguillarum 975/I.

To examine the influence of the temperature on the *empA* expression in *Vibrio anguillarum*, the strain 975\I was incubated in TSB + 1,5 % NaCl at temperatures of 15°C 25°C and 37°C. The total RNA was extracted from cells at OD_{600} 0,1, 0,6 and 0,9, corresponding to lag, exponential and early stationary phase of growth.

The relative expression levels of *empA* under experimental temperatures, compared to the optimal condition of growth, are shown in the figure 4.

The different expression levels of *empA* were calculated applying the Ct comparative method, using 25°C as calibrator and *rpoN* as housekeeping gene, during the different phases of growth.

At 15°C, the *empA* gene was over-expressed in all phases of growth tested. The calculation of $2^{-\Delta\Delta Ct}$ has shown that, during the lag phase, the expression was 3,5 fold more than at 25°C; during the exponential phase of growth was 20,89 more expressed and, finally, when the cells reached the early stationary phase the expression of target gene was 40 time more compared to expression of *empA* at 25°C.

At 37°C the data analysis showed during the lag and exponential phases an under-expression of the gene while during the early stationary phase of growth the expression of *empA* was 35 time more compared with the optimal condition of growth.

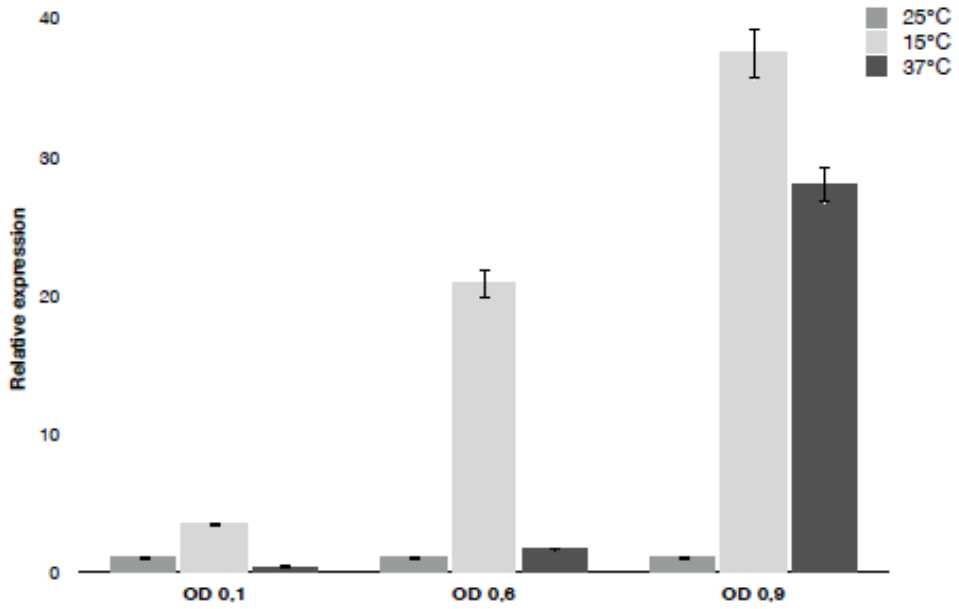


Figure 4. Differential *empA* gene expression at 15°C and 37°C compared to the standard condition of growth (25°C) in *Vibrio anguillarum* 975/I cells. x-axis shows different OD_{600} corresponding to the lag, exponential and early stationary phase of growth (respectively 10^4 CFU\ml; 10^7 CFU\ml; 10^9 CFU\ml) , y-axis shows the $2^{-\Delta\Delta Ct}$ of *empA* at the different temperatures compared to basal expression (1).

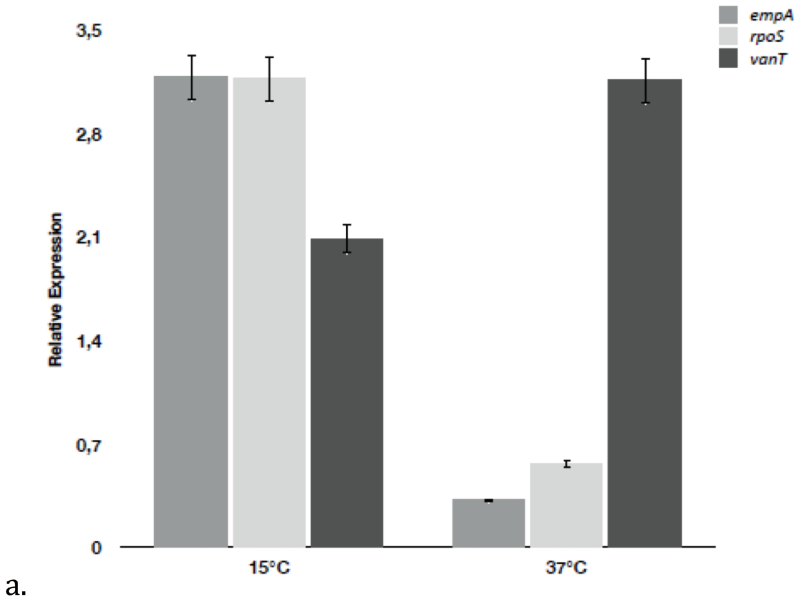
*4.3.*5 *Relative expression of empA vanT rpoS genes.*

To test if the temperature has an influence directly on *empA* gene or it has an influence on the quorum sensing process, a second set of experiments was carried out. In this case the genes analyzed with *empA* were *vanT* and *rpoS*, both involved in the quorum sensing process of *Vibrio anguillarum*.

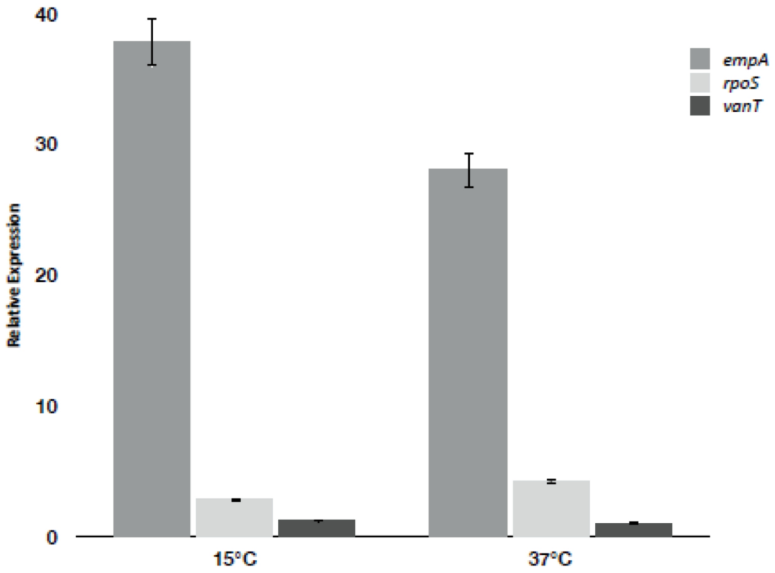
The cDNA samples used as template were the same tested during previous experiments as well as data analysis method was the same.

Figure 5 shows the differential expression levels of target genes at 15°C and 37°C during a condition of low cells density and during a condition of high cells density. Also in this case the results were normalized on the standard condition of growth (25°C) and the expression of housekeeping gene. The results show that the relative expression levels of *rpoS*, *vanT* and *empA* genes, when the cells were at low density, were almost the same for both temperature tested, 3 fold more than 25°C; when the

cells were at high density, the differential expression rates of *vanT* and *rpoS* genes weren't significantly relevant while the *empA* gene showed a high over expression; in detail the values recorded were 35 fold more at 15°C and 25 fold more at 37°C compared with the expression at 25°C



a.



b.

Figure 5. Differential expression of *empA*, *rpoS* and *vanT* genes at 15°C and 37°C during a condition of low density (a) and high density (b) compared to the standard condition of growth (25°C) in *Vibrio anguillarum* 975/I cells. Y-axis shows the $2^{-\Delta\Delta Ct}$ of target genes at the different temperatures compared to basal expression (1).

4.3.6 Influence of temperature on the EmpA protease production in Vibrio anguillarum 975\I.

In addition to examining *empA* expression at the transcriptional level, cell free supernatant was assayed with azo-casein to determine the amount of protease activity.

Cell supernatant was prepared from cells that were used to examine *empA* relative expression in figure 4.

When the cells were grown in TSB + 1,5% of NaCl at 15°C (figure 6), no protease activity was detected until a cell density of 10^7 CFU\ml, at a cells concentration of 10^9 CFU\ml the value recorded was of 5,6 Unit\ml.

Also during the incubation at 25°C and 37°C no protease activity was detected at 10^7 CFU\ml, while when the cells reach a concentration of 10^9 CFU\ml the values obtained were of 4,7 Unit\ml at 25°C and of 10,1 Unit\ml at 37°C (figure 6).

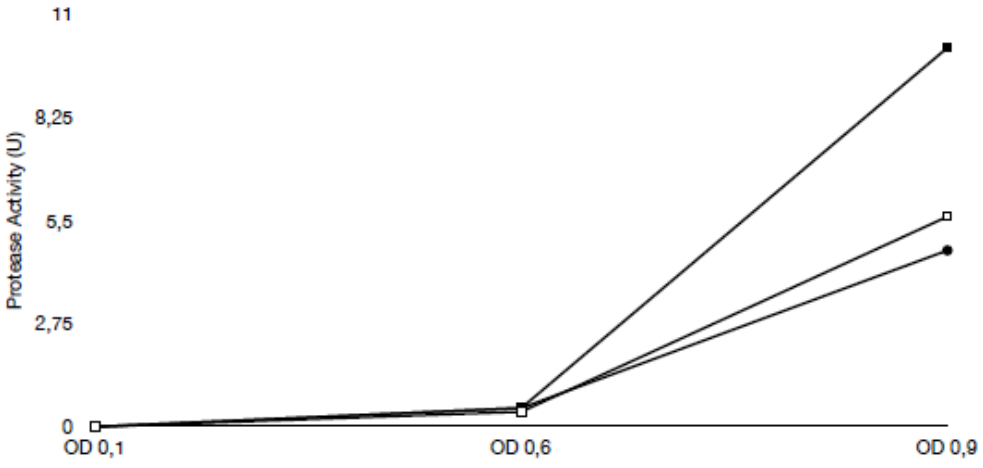


Figure 6. Protease activity in *V. anguillarum* 975/I at different cells density and temperature incubation; cells were grown 24h in TSB+1,5% NaCl, harvested by centrifugation (9000 x g, 4°C), washed twice in NSS and resuspended at 10^9 CFU\ml in fresh TSB+1,5% NaCl and re-incubated at the following temperatures: 15°C (open square); 25°C (circle); 37°C (square). Samples were taken at indicated OD_{600}, and the cell supernatant was assayed for protease activity.

Moreover, to determine the rate of protease activity induction by temperature, *V. anguillarum* cells were grown overnight in TSB + 1,5% of NaCl at 25°C; washed in NSS twice; resuspended in TSB + 1,5% NaCl fresh media at 10^9 CFU\ml and incubated either at 15°C, 25°C and 37°C.

The data showed in the figure 7 reveal that protease activity was induced within 90 min after the incubation at 15°C and 37°C; therefore the induction at 25°C was activated after 180 min of incubation. Protease activity continues to increase over the following 2 hours. The maximum value recorded at 15°C was 22,5 Unit\ml.

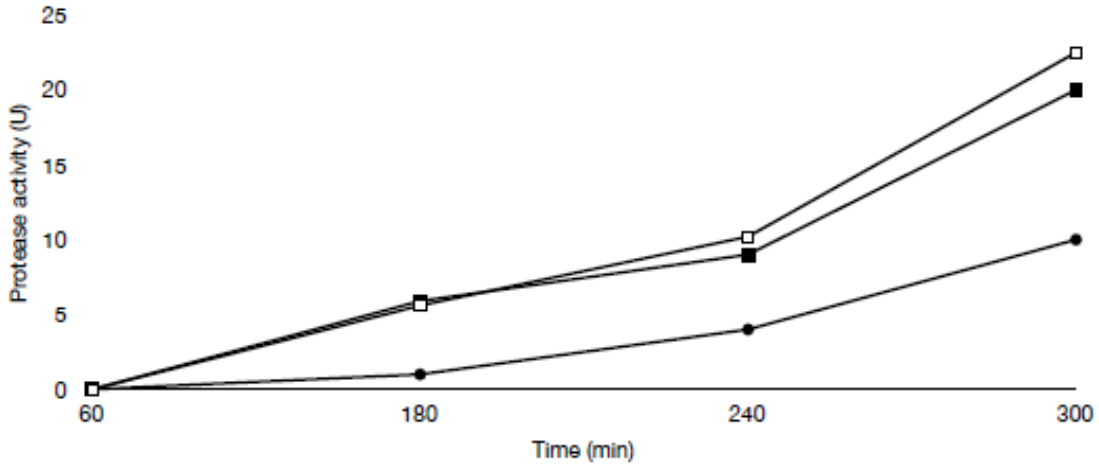


Figure 7. Induction of protease activity in *V. anguillarum* 975/I by temperature; cells were grown 24h in TSB+1,5%NaCl, harvested by centrifugation (9000 x g, 4°C), washed twice in NSS and resuspended at 10^9 CFU\ml in fresh TSB+1,5% NaCl and re-incubated at the following temperatures: 15°C (open square); 25°C (circle); 37°C (square). Samples were taken at indicated time incubated times, and the cell supernatant was assayed for protease activity.

4.3.7 influence of growth media on empA expression.

To test the influence of growth media on the *empA* trascription, total RNA was extracted from *V .aguillarum* cells grown in TSB + 1,5% of NaCl, cM9 and ONR7a (both supplemented with 0,2% of Casamino acidis and 0,5% of Glucose); the incubations were carried out at 25°C for 24h (until to reach the concentration of 10^9 CFU\ml). The

samples collected were used as a template for the real time PCR assays; the results showed that there wasn't an appreciable influence of different media on *empA* expression, the $2^{-\Delta\Delta Ct}$ values were almost the same for all media tested, the differential expression of *empA* was constant. (data not shown).

4.3.8 Influence of growth media on the EmpA protease production in Vibrio anguillarum 975\I.

In addition to examining *empA* expression at the trascriptional level, cell free supernatants of cells grown in several culture media were assayed with azo-casein to determine the amount of total protease activity.

Cell supernatant was extracted from *Vibrio anguillarum* 975\I cells grown in 3 different growth media, TSB, cM9 and ONR7a for 24h at incubated either at 15°C, 25°C and 37°C.

The data analysis are showed in the figure 7.

The results showed that exist a significantly different of the protease activity for the growth media tested. In detail the minimum value of activity was detected in the supernatant collected form cells grown in ONR7a; in TSB + 1,5% of NaCl the values recorder were of 5,6 Unit\ml at 15°C, 3,9 unit\ml at 25°C and 10,1 unit\ml at 37°C. The highest values of activity were detected in supernatants collected from cells incubated in cM9 with a maximum values of activity measured at 15°C of 124 unit\ml

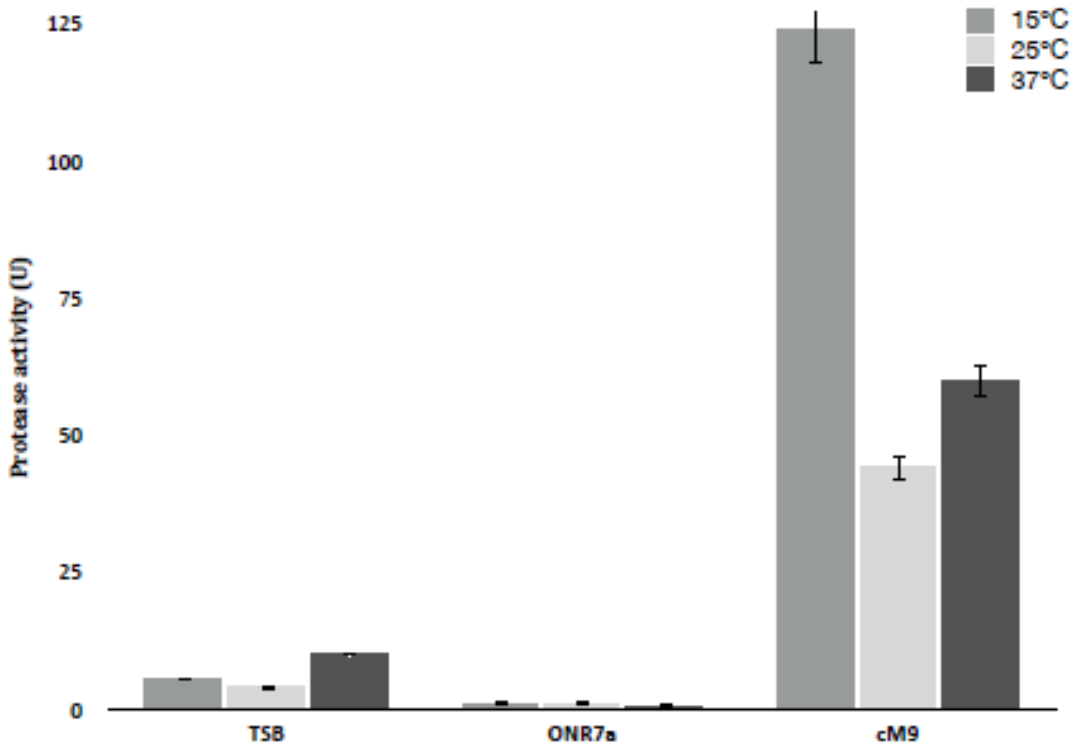


Figure 8. Protease activity in *Vibrio anguillarum* 975/I incubated in TSB, ONR7a and cM9.
Samples were taken after an incubation of 24h at different temperature (15°C, 25°C, 37°C)., and the cell supernatant was assayed for protease activity.

Moreover, to obtain a specific detection of EmpA proteases activity, western blot analysis was carried out on using an antiserum against EmpA. Cell free supernatants analyzed were the same used for the azo-casein assay in figure 8.

Western blotting revealed two reactive bands at 46KDa, and 38kDa corresponding respectively to the pro-EmpA and the mature EmpA. These two bands were present only in two samples, in the supernatant of cells grown in cM9 at 15°C and 25°C; at 37°C for the same media was present only the active form of EmpA (38 kDa). For TSB was visible only the 46kDa band at 37°C therefore for ONR7a the 46 kDa was visible

at 15°C; no more bands were detected. (Figure 9).

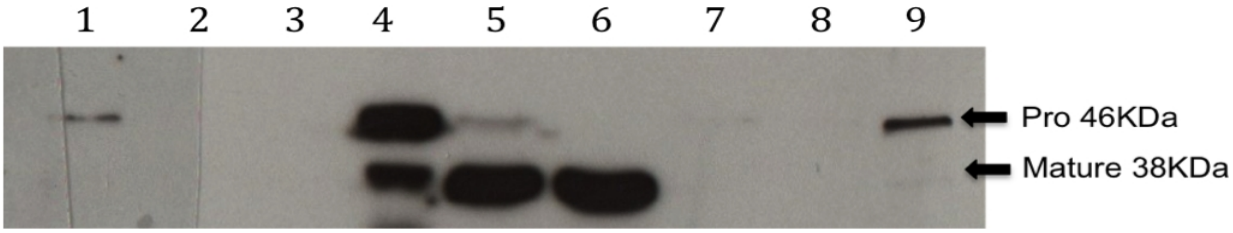


Figure 9. Western blot analysis of EmpA secretion of *V.anguillarum* 975/I at the cells density of 10^9 CFU\ml grown in ONR7a, cM9 and TSB at the temperature of 15°C, 25°C and 37°C. Lane 1, supernatant from 975/I in ONR7a at 15°C; line 2, supernatant from 975/I in ONR7a at 25°C; line3, supernatant from 975/I in ONR 7a at 37°C; line 4, supernatant from 975/I in cM9 at 15°C; line 5, supernatant from 975/I in cM9 at 25°C; line 6, supernatant from 975/I in cM9 at 37°C; line 7 supernatant from 975/I in TSB at 15°C; line 8, supernatant from 975/I in TSB at 25°C; line 9 from 975/I in TSB at 37°C. The two bands visible correspond with the pro-form (46 KDa) and the mature-form (38 KDa) of EmpA.

4.3.9 Protease induction by growth media.

The result of growth experiments demonstrated that stationary-phase, cells grown in cM9 were strongly induced to express EmpA protease. To determine the rate of protease activity induction by growth media, *Vibrio anguillarum* cells were incubated overnight in TSB at 25°C; washed twice in NSS; resuspended in either TSB, cM9 or in supernatant collected from cells grown in cM9 (after incubation overnight) until to reach a concentration of 10^9 CFU\ml; and allowed incubated at 25°C. Samples were withdrawn and assayed for protease activity periodically. The data presented in figure 10 revealed that protease activity was not induced significantly in cell resuspended in TSB for 4h, moreover is clear that the protease activity was induced in the same cells incubated in cM9 after 3 h, the value obtained after 4 hours was 110 Unit\ml. Therefore the cells incubated in the supernatant collected from cells grown overnight in cM9 showed the highest induction within 90 min, protease activity continued to increase over the following 3 h until to reach a value of 469 Unit\ml.

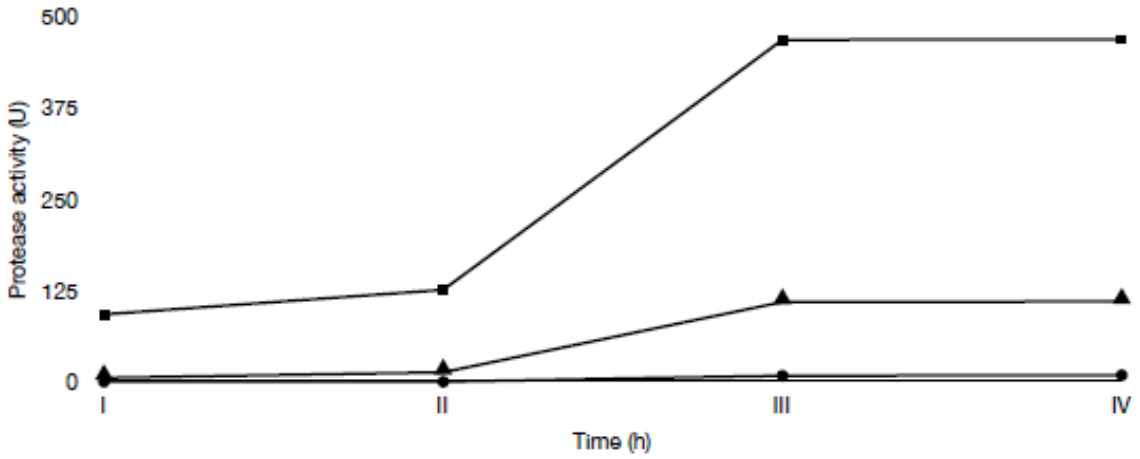


Figure 10. Induction of protease activity by growth media in *V.anguillarum* 975/I; cells were grown 24h in TSB+1,5%NaCl, harvested by centrifugation (9000 x g, 4°C), washed twice in NSS and resuspended at 10^9 CFU\ml in fresh TSB+1,5%NaCl (circle); in fresh cM9 (triangle) and in supernatant of *Vibrio anguillarum* 975/I pure culture incubated in cM9 for 24h at 25°C (square). Samples were taken at indicated time incubated times, and the cell supernatant was assayed for protease activity.

4.3.10 Western blot analysis to test the influence of NaCl on EmpA protease production.

To determine whether if the difference in EmpA protease activity in different concentration of NaCl were due to differences in translation of message or posttranslational secretion and modification, Western blot analysis was performed. Cultures of *Vibrio anguillarum* were grown to stationary phase (24h) in cM9 with 3 different concentration of NaCl. The concentration chosen were corresponding to 0,5%, 1,5% and 3% of NaCl concentration present, respectively, in cM9, TSB and ONR7a (fig 11). Western blotting revealed, at the concentration of 0,5% and 1,5% of NaCl, two band in culture supernatants with estimated molecular masses of 46 kDa and 38 kDa, representing pro-EmpA and mature EmpA; moreover at the concentration of 0,5% both bands were showed higher intensity than the two bands detected at 1,5% of NaCl. In culture supernatant of cells incubated in cM9 with a final concentration of 3% of NaCl only one band was detected with estimated molecular

mass of 38 kDa corresponding to the mature form of EmpA.

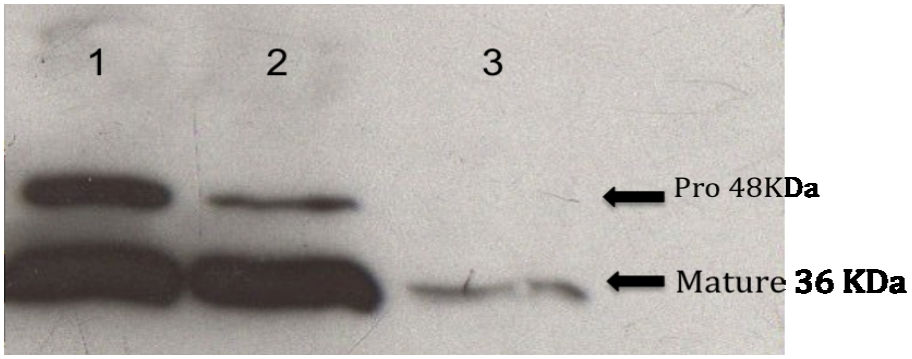


Figure 11. Western blot analysis of EmpA secretion in *V.anguillarum* 975/I at the cells density of 10^9 CFU\ml grown in cM9 with a final concentration of NaCl of 0,5% (line 1), in cM9 with a final concentration of NaCl of 1,5% (line2) and in cM9 with a final concentration of NaCl of 3% (line 3). The two bands visible correspond with the pro-form (46 KDa) and the mature-form (38 KDa) of EmpA.

4.4 Discussion

The aim of this work was to verify the effect of environmental changes on the expression of the *empA* gene in *Vibrio anguillarum*, encoding for a metallo-protease, and the translation of the product.

We tested temperature and salinity as typical factors affecting life and adaptive response in microorganisms, with major emphasis in pathogens which have to recognize inter-host environment and take advantage from it. These relate to the environmental risk for farmed and wild-living fish which can inhibit or stimulate pathogens, as *Vibrio anguillarum,* favoring outbreak diseases (Austin and Austin 1987; Bolinches *et al.* 1986; Bowser *et al.* 1981; Mizuki *et al*. 2006; Mihaljevic *et al.* 2007; Taranzo and Barja 1993).

Experiments were carried out *in vitro* although not always represent the real behavior of microorganism, they often help the comprehension of the expression machinery potential on the feedback to specific environmental stimuli. As an example, recent studies have demonstrated that Vibrio anguillarum produces EmpA protease *in vitro*

when the growth medium is supplemented by intestinal mucus simulating the host environment (Denkin and Nelson 1999).

Growth rates (together with the viability) show that, regardless of culture medium, 15°C seems to be a stressful condition. From the literature it is evident that temperature is a critical factor for the growth of vibrios (Kaspar and Taplin 1993; Covert and Woodburn 1972; Ben-Haim *et al.* 2003; Randa *et al.* 2004; Soto *et al.* 2009; Larsen 1984), which results in a characteristic annual cycle with the highest frequencies coinciding with the highest temperatures. The tolerance of temperature variation by *Vibrio anguillarum* strains depends on geographic areas but also on their own temperature optimum (Conchas 1991; Serensen and Larsen 1985; Larsen *et al.* 1988). In our case, *V. anguillarum* 975/I shows the highest growth performance at 37°C while the most stressful condition was 15°C when we have coupled the increasing of NaCl amount up to 2.9%, such values of temperature and salinity are typical parameters of Mediterranean sea (from which 975/I strain was isolated).

The analysis of the expression of *empA* put in evidence that, the response of *V. anguillarum* to temperature variations was different at 15°C and 37°C. In fact, during the incubation at 15°C, we detected an increase in expression of *empA* since the early phase of growth that enhanced according to the cells density.

At 37°C we were able to detect a drastic shift on *empA* expression only when the cells reached the concentration of 10^8 CFU\ml, this result agree with the model of quorum sensing process in *Vibrio anguillarum* proposed by Weber *et al.* (2008).

This scenery was common to all media tested (TSB, cM9 and ONR7a).

Moreover, interesting ,we observed a differential behavior on the translation of the proteases in fact the inducer effect of salinity (tested in cM9 medium) occurs only at

0.5 % NaCl (very similar to that one within the host at blood tissue level).

This confirms that the perception of environmental variation by *Vibrio anguillarum* could have a prompt reaction in the expression of mRNA (with a selective increasing of specific gene copy number) but there is a translational regulatory network which determines the success of the protein production.

The results of the present study have an environmental key lecture, osmotic stress could be one of the factors which promotes and drives the preliminary phase of infection: the approach to the host and adhesion to the skin. With special attention to EmpA production, this result suggests that additional environmental parameters could amplify the effect of high cell density condition during quorum sensing process.

Further studies are required to verify the involvement of Epp protease on the post-transcriptional control following the environmental stimuli tested in our study (Staroscik *et al.* 2005).

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Chapter 5

"Comparison between *16SrDNA* and *toxR* genes as target for the detection of *Vibrio anguillarum* 975/I in *Dicentrarchus labrax* Kidney and liver."

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5 Comparison between *16SrDNA* and *toxR* genes as target for the detection of *Vibrio anguillarum* 975/I in *Dicentrarchus labrax* Kidney and liver.

5.1 Introduction.

Vibrio anguillarum is an opportunistic fish pathogen frequently detected in marine and estuarine environments (Austin and Austin, 1987; Egidius, 1987; Sinderman, 1990; Thompson *et al.* 2004; Toranzo *et al.* 1987). It has been identified as one of the main cause of vibriosis, a disease that leads to great economic losses in fish farming worldwide.

Rapid identification of pathogens is crucial for effective disease control in aquaculture; detection of pathogens is important not only in infected fish (clinically and sub-clinically), but also in the environment e.g. between harvesting and re-stocking, and as an 'early warning system'.

The phenotypic determination and taxonomic identification of *V. anguillarum* in the complex microbial community inhabiting fish tissue require specific target for a rapid and reliable diagnosis.

Isolation of *V. anguillarum* generally is performed in selective medium containing 1 to 2% NaCl, mainly thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Bolinches *et al.* 1988; Alsina *et al.* 1994; Farmer *et al.* 1992), which seems to be specific for *Vibrio* genus, for this reason it is not indicated for the specific identification of *V. anguillarum*.

Today, the molecular methods overcome the battery of biochemical and serological tests, but such methods require the subculture of numerous single isolates and phenotypic variability of strains could make identification questionable; routine

processing is thus time consuming and expensive, with poor or low levels of sensitivity.

An emerging method for the detection and identification of a variety of infectious agents in clinical laboratories is the real time PCR (Espy *et al.* 2006; Laverick *et al.* 2004). This modification of the traditional PCR assay was developed to improve the sensitivity, specificity, and speed of detecting PCR amplification products. Moreover this method is about 100 times more sensitive and faster than conventional PCR (Lyon, 2000; Campbell and Wright, 2003). Although real time PCR assays are based on the amplification of *16S* and *23SrRNA* genes, which are found in all eubacteria, there is a high degree of genetic similarity for these genes across taxa (Gonzalez *et al.* 2003; Arias *et al.* 1995; Hoie *et al.* 1997; Magnusson *et al.* 1994; Marshall *et al*. 1998; Osorio *et al.* 1999); therefore, the specificity of the detection method can be compromised (Kita-Tsukamoto *et al.* 1993; Ruimy *et al.* 1994). Alternatively, is possible to choose specific genes (e.g., virulence loci) that can be used as targets for PCR amplification to permit more specific detection (Gonzalez *et al.* 2004; Osorio and Toranzo, 2002).

In the present study, we have evaluated the efficiency of real time PCR on the specific detection and quantification of *V. anguillarum* in fish tissue. Together with *16SrDNA*, frequently used as target for identification of bacterial strains, we have tested *toxR* gene as alternative specific target. Moreover analyses were conducted after injection of 50% lethal dose to assess the bacterial load of *V. anguillarum* in target tissue.

5.2. Materials and Methods

5.2.1 Bacterial strains and Media.

The bacterial strain used in this study was *V. anguillarum,* serotype O1 975/I a local

(Mediterranean Sea) virulent strain (kindly provided by Dr. Manfrin, Istituto Zooprofilattico delle Venezie-Italy) isolated from *sea bass* specimens.

The strain was routinely cultured in Tryptic Soy Broth (Difco: Pancreatic Digest of Casein 17.0 g\l, Enzymatic Digest of Soybean Meal 3.0 g\l, Sodium Chloride 5.0 g\l, Dipotassium Phosphate 2.5 g\l, Dextrose 2.5 g\l supplemented with 1% w\v of NaCl) at 25°C and on Thiosulfate Citrate Bile Sucrose Agar (TCBS agar Difco: Yeast Extract 5.0 g\l, Protease Peptone No. 3 10.0 g\l, Sodium Citrate 10.0 g\l, Sodium Thiosulfate 10.0 g\l, Oxgall 8.0 g\l, Saccharose 20.0 g\l, Sodium Chloride 10.0 g\l, Ferric Ammonium Citrate 1.0 g\l, BromthymolBlue 0.04 g\l ThymolBlue 0.04 g\l Agar 15.0 g\l supplemented with 0.5% w\v NaCl) at 25 °C. All the incubations were carried out under moderate agitation (150 rpm).

5.2.2 Bacterial growth and samples collection.

The bacterial growth was monitored by a biophotometer (Eppendorf BioPhotometer) at $O.D._{600nm}$. At the O.D. values of 0.1, 0.6, 1, corresponding to lag, exponential and early stationary phase, aliquots of the broth culture were collected for CFU count and for real time PCR analyses. Samples, showing $O.D._{600nm}$ 0.6 and corresponding to 10^6 CFU ml^{-1}, were utilized for fish infection.

5.2.3 CFU count in pure culture and infected tissue.

Aliquots of 100 µl of pure culture 10-fold serially diluted were spread on sterile Tryptic Soy Agar (TSA) (Oxoid) and incubated at 25°C for 48 h. Only plates showing CFU between 30 and 300 colonies were counted.

An equal quantity (0.01g) of fish tissue (kidney, liver and muscle) from infected and uninfected fish was homogenized in Phosphate buffered saline (PBS) pH 7,4 with a tissue lyser homogenizer (Qiagen); 100 µl of homogenates were spread on sterile TSA

media and processed as indicated above.

5.2.4 DNA extraction from bacterial cells.

DNA was extracted from 2ml of *Vibrio anguillarum* pure culture collected at specific $O.D._{600}$ 0.1, 0.6, 1 using Qiagen RNA/DNA Mini Kit. The cells were harvested by centrifuge at 5000-x g for 10 min at 4 °C and treated according to the manufacture's instructions.

DNA was stored in isopropanol at –20ºC before the precipitation. The DNA was resuspended in 50 µl of RNase free water.

The quality and concentration of the samples were determined using the NanoDrop® ND-1000 Spectrophotometer (Celbio).

The DNA was used as template for further real time PCR amplifications.

5.2.5 DNA extraction from animal tissue.

Samples of 0,01 g of each organ were firstly treated with 20 µl of protease K, the mixture was incubated at 55°C and then DNA was extracted from tissue (kidney, liver and muscle) using Qiagen DNeasy® blood & tissue kit (Qiagen) according to the manufacture's instructions, with minor modification: the incubation time for lyses was extended overnight.

The quality and concentration of the DNA samples were determined using the NanoDrop® ND-1000 Spectrophotometer (Celbio).

The DNA extracted was used as a template for further real time PCR amplification.

5.2.6 LD_{50}.

With the aim to verify the virulence of the strains 975/I, LD_{50} was performed.

Before the infection, *Vibrio anguillarum* cells were prepared by growing in Marine broth for 24 h at 24 °C to a density of $\cong$ 2 x 10^9 CFU/ml, then, bacterial suspension

was spread in TCBS agar plates added with 1,5% of NaCl (final concentration) and in Marine agar plates for 24 h at 24°C. Grew colonies were isolated in pure culture and identified.

Successively an aliquot was diluted into 10 ml of marine broth up to an $OD_{600\ nm}$ 0.1 and then centrifuged at 5000 x g to collect the pellet that was re-suspended into saline sterile solution.

Aliquots of 0.1 ml of bacterial suspension were intraperitoneally injected into 10 specimens of *D. labrax* (mean weight 20 g).

Dead fish were dissected and the infected kidneys were spread on TCBS agar plate for the isolation of *Vibrio anguillarum* strains. This operation was repeated three times.

After this treatment, 100 µl of the bacterial suspension of *Vibrio anguillarum* at different concentrations: 10^3 cell ml^{-1}; 10^4 cell ml^{-1}; 10^5 cell ml^{-1}; 10^6 cell ml^{-1}; 10^7 cell ml^{-1}, (Litchfield and Wilcoxon, 1949) were injected intraperitoneally into 80 fish (m.w. 20 g). Fish, used as control, were inoculated with 100 µl of sterile saline solution.

Temperature and salinity were maintained respectively at 24°C and 38 ‰ in 100 L water flow tanks under natural photoperiod.

5.2.7 Fish Infection.

Aliquots of 0,1 ml of *V. anguillarum* suspension *(*10^6 cells ml^{-1}) were injected intraperitoneally (i.p.) into 50 specimens of *D. labrax* (m. w. 20 g), divided in 5 groups and 0.1 ml of sterile saline solution were inoculated in 20 specimens used as control. After 18 h, fish (from both infected and control) were sacrificed and kidney, liver and muscle were extracted.

5.2.8 Primers and specificity.

The primers set specific for the *16SrDNA* and *toxR* genes of *V. anguillarum* was newly

designed in this study using Primer Express software (Primer Express software, version 2.0 (Applied Biosystems, Foster City, Calif.) with reference to the partial sequence of *V. anguillarum* published in GeneBank (16SrDNA AY069970, ToxR AJ299739). The sequences of the primer sets used for real time PCR analysis are shown in Table 1.

The specificity of the amplified fragments was tested against the strains described in Table 2 and in a mixed sample of DNA (from pure culture in a background of tissue extract). Defined amounts of DNA extracted from *V. anguillarum* (0.1 and 0.001 ng) were spiked into tissue homogenates extract, and used as template for PCR with specific primers. The sequences of fragments were checked by BLAST search in the GenBank database (**http://www.ncbi.nml.nih.gov**)

Table 1: Primers used in this study. The couples of primers write in bold were used in the further analysis.* Melting temperature is calculated by Primer Express software version 2.0.
** Optimal combination of primers concentration detected by Real Time PCR.

Gene/Primer	Sequence Forward (5'-3')	Sequence Reverse (5'-3')	Amplicon length (bp)	Melting Temperature (°C)*	F/R (nM)**	Source of reference
16S rDNA	**CCACGCCGTAACGATGTCTA**	**CCAGGCGGTCTACTTAACGCGT**	**81**	**82**	**50/100**	**In this study**
16S1 rDNA	TGCCAGCGAGTCATGTCG	CGTAAGGGCCATGATGACTTG	91	81	100/100	In this study
16S2 rDNA	AGCACCGGCTAACTCCGTG	TGCGCTTTACGCCCAGTAAT	81	82	100/50	In this study
16S3 rDNA	CCTGGTAGTCCACGCCGTAA	CCAGGCGGTCTACTTAACGC	91	81	50/50	In this study
toxR	**ACACCACCAACGAGCCTGA**	**TTGTCTCTTCGGGTTGCGA**	**93**	**80**	**100/50**	**In this study**
toxR1	CACCACCAACGAGCCTGAA	TTGTCTCTTCGGGTTGCGA	92	80	100/100	In this study
toxR2	GCCTGAAGAGGAACCGTTACTG	CTGTCGTCACGGTTTGGGAT	101	81	50/100	In this study
toxR3	GAGCCTGAAGAGGAACCGTTAC	CTGTCGTCACGGTTTGGGAT	103	81	50/50	In this study
27F\1492R	AGAGTTTGATCCTGGCTCAG	GGTTACCTTGTTACGACTT	1465	//	//	Lane 1991

5.2.9 Real-time PCR and cycling parameters

To find the primer concentrations that would produce the optimal amplification signal, nine different combinations between 50 and 900 nM of forward and reverse primers were tested (Table 1).

Real Time PCR was run in a 7300 Real Time PCR System (Applied Biosystems, Foster City, Calif.) in triplicate. Reaction mixture was prepared using SYBR® Green PCR Master Mix Applied Biosystems in a total volume of 25 µl containing 12,5 µl of Master Mix, 0,5 µl of primers forward and reverse (at optimized concentrations), 1 µl of DNA containing 20ng from pure culture samples and 50ng from tissue samples. Sterile MilliQ water was used to adjust the volume of each reaction to 25 µl.

A no-template control (NTC) and a positive control DNA from pure culture were included on each plate.

The thermal cycling protocol included an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing/elongation at 60°C for 60 s. Dissociation step was added to check for primer-dimer formation.

5.2.10 Construction of standard curves for 16SrDNA and toxR copy number determination.

A ten-fold serial dilution series, ranging from 10 to 10^8 number of cells of *V. anguillarum* per reaction, was used to create the standard curve for the quantification. Serial dilutions were prepared once for both targets and used for real time quantification. The concentration of DNA was measured using NanoDrop® ND-1000 Spectrophotometer (Celbio) and converted to the copy concentration using the following equation (Whelan et al., 2003):

DNA (copy) = 6.02×10^{23}(copies mol^{-1}) × DNA amount (g) / DNA length (*V. anguillarum* 4.2 Mbp) ×660(g $mol^{-1}bp^{-1}$) (Rodkhum et al., 2006).

Standard dilutions were analyzed in triple. The Ct values were plotted against the logarithm of their initial template copy concentration. Standard curves were generated by a linear regression of the plotted points. From the slope of each curve,

PCR amplification efficiency (E) was calculated according to the following equation:

$E = 10^{-1/\text{slope}} - 1$.

5.3 Results

5.3.1 LD_{50}.

With the aim to assess the virulence of *Vibrio anguillarum* strain 975/I in terms of mortality of fish, LD_{50} measurements have been applied, after intraperitoneal injection. The time for the development of the illness was directly related to the bacterial load of the inoculum, namely, fish infected with a bacterial load of 10^6cell ml^{-1} and 10^7cell ml^{-1} displayed clinical sign after 18h (hemorrhages in the basal fin and mouth and caudal erosion, others showed only internal hemorrhages), the same was observed after 48h with a load of 10^5cell ml^{-1}, whereas no clinical sign were observed (within the 48h) in fish infected with an inoculum of 10^4 cell ml^{-1} and 10^3cell ml^{-1}. The mortality was monitored twice a day and, within the 48h, the lethal dose causing the mortality of the 50% of the infected fish was 10^6cell ml^{-1}. Further challenge and molecular analyses were performed using an inoculum of 10^6cell ml^{-1}.

5.3.2 Primers design and specificity.

DNA from pure cultures of *Vibrio anguillarum* was successfully PCR-amplified with all tested primers (table 1) whereas DNA extracted from fish tissue was amplified with only a single primer pair (indicated in bold in table 1 and used for further analyses). Figure 1a-b shows the specificity of the corresponding amplification products observed during a melting curve analysis between 60°C and 95°C, only specific amplification showed a single peak at the expected temperature, namely 81°C for *16SrDNA* target and 80°C for *toxR*.

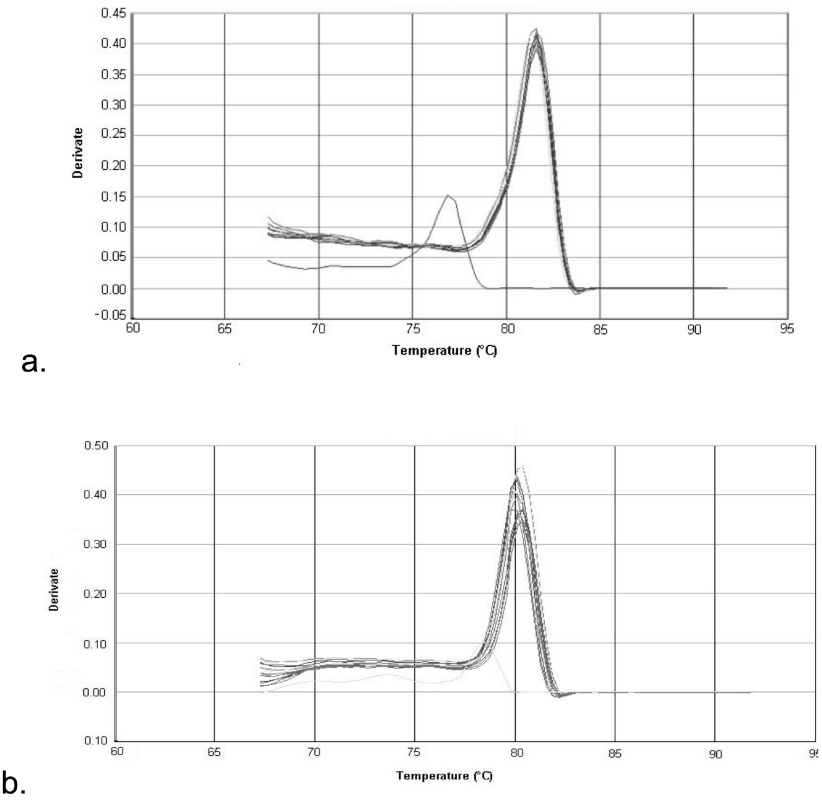

Figure 1 a-b. Dissociation curves analysis using 16SrDNA primers (melting temperature 81°C) (a) and ToxR primers (melting temperature 80°C) (b). The dimers detected in both curves corrspond to the no-template control.

The test for the optimization of primers concentrations (table 1) showed that the most appropriate primers combination was 50/100 nM forward/reverse for *16SrDNA* and 100/50nM for *toxR*, at which the lowest threshold cycle was detected. The specificity of the primers was also tested using several species of bacterial groups (table 2), no significant cross-reaction was observed among tested strains other than *V. anguillarum.*

The spiking experiments were performed to test the ability of this assay to pick out a specific bacterial DNA from a complex background of DNA (i.e., extracted from tissue specimens) and to investigate any negative interference, as inhibition, in the amplification reaction. Results observed on 2% agarose gel showed a specific amplification fragment whose fluorescence level was about 2-5% lower than

amplicons from pure cultures (data not shown). Moreover, tissue DNA was efficiently amplified with universal primers 27F/1492R targeted to the conserved region of bacterial 16SrDNA, as internal amplification control.

The identities of the PCR amplicons from the isolates and tissue were confirmed by sequencing.

The sequence data showed that *V. anguillarum* strains contained 100% identical nucleotides over the fragments investigated, as the percent identity of fragments obtained by tissue ranged from 97 to 98%.

5.3.3 Real-Time PCR sensitivity.

In order to verify the sensitivity of the method and the consistency of the quantitative measurement, DNA purified from a pure culture of *V. anguillarum* sampled at O.D. $_{600}$ 1 was serial-diluted (10-fold dilutions) and analyzed in real time PCR.

Figure 2 shows standard curves of *16SrDNA* and *toxR* genes. From the average of Ct values SyBR Green assay is exponential over abroad dynamic range, from 10 to at least 10^8 CFU starting material.

The slopes values, namely, -3.17 for *16SrDNA* and -3,29 for *toxR,* fall within the optimal range, corresponding to the efficiency of 99.6 and 96.2% respectively. The correlation within the optimal range as well (r= 0.99).

Table 2: Bacterial strains used to test the sensitivity and specificity PCR for the identification of *Vibrio anguillarum.* (ATCC, American Type Culture Collection) **(CGMCC The China General Microbiological Culture Collection Center.)

Species	Strain(s)	Gene(s) in study			
		16S rDNA	***16S1 rDNA***	***toxR***	***toxR1***
Vibrio adaptus	ATCC 19263^{T}	-	-	-	-
Vibrio aestuarianus	ATCC 35048^{T}	-	-	-	-
Vibrio alginolyticus	ATCC 14582^{T}, 17749^{T}, 19108^{T}	-	-	-	-
Vibrio anguillarum					
serotype O1	975\I , 775 , 531	+	+	+	+
serotype O2	ATCC 43306^{T}	+	+	+	+
serotype O3	ATCC 43307^{T}	+	+	+	+
Vibrio costicola	ATCC 33508^{T}	-	-	-	-
Vibrio fisheri	ATCC 7744^{T}, 33983^{T}	-	-	-	-
Vibrio furnissii	CGMCC 1.1613	-	-	-	-
Vibrio logei	ATCC15382^{T}	-	-	-	-
Vibrio marinagilis	ATCC 14398^{T}	-	-	-	-
Vibrio mediterranei	ATCC 43341^{T}, 43342^{T}	-	-	-	-
Vibrio ordalii,	ATCC 33509^{T}	-	-	-	-
Vibrio pelagius	ATCC 25916^{T}, 33504^{T}, 33782^{T}	-	-	-	-
Vibrio ponticus	ATCC 14391^{T}	-	-	-	-
Vibrio splendidus	ATCC 33125^{T}	-	-	-	-
Vibrio vulnificus	ATCC 27562^{T}, 33147^{T}	-	-	-	-
Listonella pelagia	ATCC 25916	-	-	-	-
Photobacterium damselae subsp. *damselae*	ATCC 33539	-	-	-	-
Photobacterium damselae subsp. *piscicida* strains	ATCC 17911^{T}	-	-	-	-
Lactococcus garvieae	ATCC 43921^{T}	-	-	-	-
Rhodococcus erythropolis	ATCC 25544	-	-	-	-
Aeromonas salmonicida subsp *salmonicida*	ATCC 33658T	-	-	-	-
Aeromonas hydrophila	ATCC 7966^{T}	-	-	-	-
E. coli	ATCC 8739^{T}	-	-	-	-

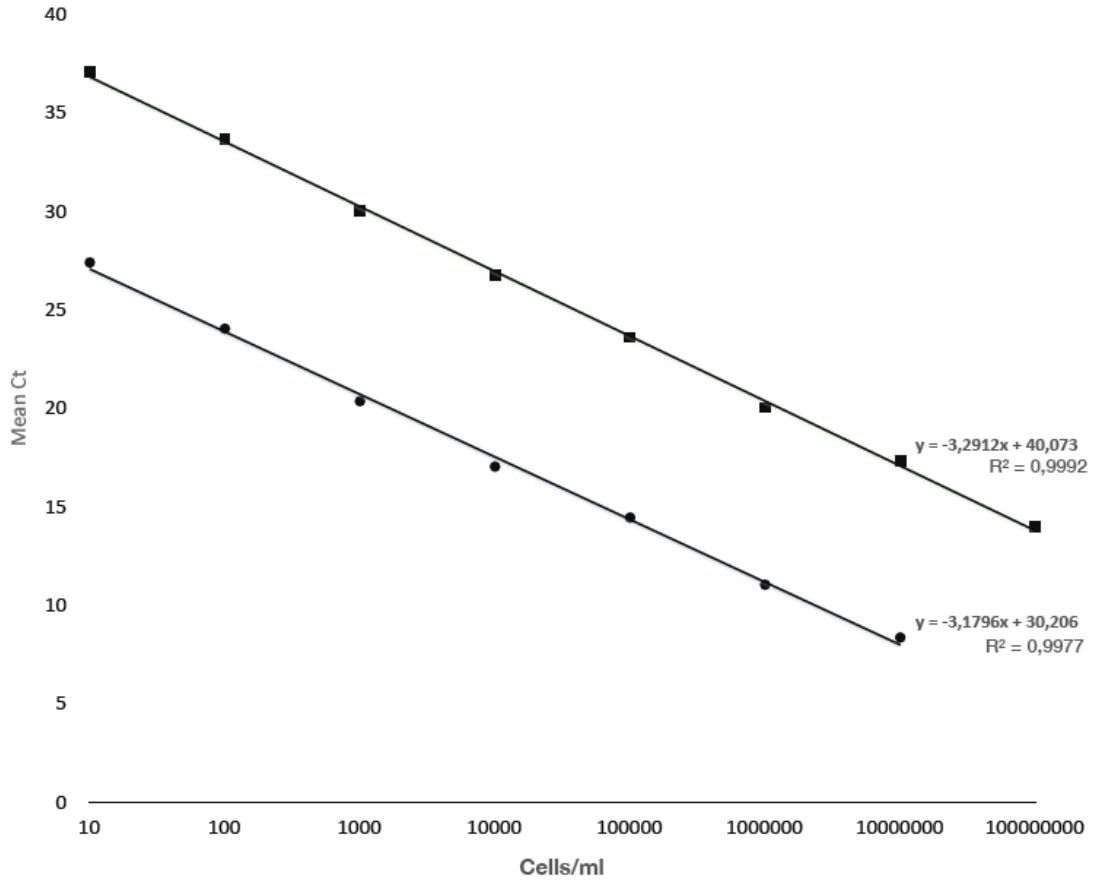

Figure 2. Standard curves for quantification, plotted from triplicate samples using Ct values of tenfold dilutions of DNA template extracted from *V. anguillarum* 975\I. *16SrDNA*(circle) *toxR* (square).

Results of the quantitative assay show that, within each sample (in triplicate) the ratio between copy number *16SrDNA*/copy number *toxR* detected was approximately around 9.40 and the number of the genomic target *16SrDNA* resulted one order of magnitude higher than the quantity of copies detected for *toxR*. The same relation was consistent with each point of the standard curves and with the other samples tested in the following assays, both in pure culture and tissue (table 3 and 4).

5.3.4 Quantification on pure culture.

The efficiency of the quantification methods was also investigated on pure culture collected at specific OD_{600} corresponding respectively to the early exponential and early stationary phases. The quantity of DNA extracted from 1 ml of pure culture at

the OD_{600} 0.1, 0.6, 1 was respectively 20 ng μl^{-1}, 157 ng μl^{-1} and 553 ng $\mu l^{-1}$and the number of cells detected during the growth phases (table 3) was respectively 10^6, 10^7, 10^8 cells ml^{-1} which was one order of

magnitude higher than CFU count on TCBS plate.

Table 3: Quantification of *Vibrio anguillarum* 975\I from the pure culture in Tryptic Soy Broth with a final concentration of 1.5% NaCl.

Vibrio anguillarum **(O.D. 600_{nm})**	**Colony-Forming Units (CFU ml^{-1} ± SD)***	**Num. Cells ml^{-1} ± SD Real Time PCR****	**Copy number 16S rDNA ng^{-1} ± SD*****	**Copy number toxR ng^{-1}± SD*****
0.1	3.3×10^5 ± 0,07	3.1×10^6 ± 0,02	1.3×10^6 ± 0,09	1.4×10^5 ± 0,06
0.6	1.3×10^6 ± 0,08	2.6×10^7 ± 0,01	0.93×10^6 ± 0,08	0.98×10^5 ± 0,09
1	0.8×10^8 ± 0,10	4.1×10^8 ± 0,02	1.2×10^6 ± 0,10	1.3×10^5 ± 0,13

*V. anguillarum levels were determined by plate counts of CFU on Tryptic Soy Agar with a final concentration of 1.5% NaCl spread plates.
**Real-time PCR determination of V. anguillarum cells per ml of pure culture was based on the mean of triplicate samples. Concentrations were derived from a standard curve using the mean of triplicate Ct values for serial tenfold dilutions of DNA extracted from known concentrations of bacteria.
***Real-time PCR determination of genes copy number per ng of DNA of V.anguillarum.

5.3.5 Quantification on infected fish.

Table 4 reports the number of cells detected by real time PCR in tissue compared to the detection by cultural methods. After 18 h from the infection with 10^6 cell ml^{-1} the relative density of *V. anguillarum* cells averaged around 10^6 cell gr^{-1} of tissue. Although the amount of genomic targets was similar in all tissue, kidney samples showed a quite higher quantity of cells than the liver samples. The minimum quantity appreciable by real time PCR in fish organs was 2×10^2 to 2×10^3 cell g^{-1} tissue detected on muscles. Pathogens usually colonize firstly well-vascularized tissues as gills, kidney, liver, spleen, and heart, this could explain the lower amount of cells detected in muscles. Also in this case CFU count was one order of magnitude lower than real time quantification.

Table 4: Quantification of *Vibrio anguillarum* 975\I in *Dicentrarchus labrax* tissue

Groups of infected fish*	Part(s)	Copy number *16S rDNA* gr^{-1} tissue ± SD***	Copy number *toxR* gr^{-1} tissue ± SD***	Colony-Forming Units (CFU) gr^{-1} tissue ± SD**
I	Kidney	$2.3 \times 10^7 \pm 0.09$	$2.4 \times 10^6 \pm 0.12$	$2.4 \times 10^5 \pm 0.11$
	Liver	$1.2 \times 10^7 \pm 0.14$	$1.5 \times 10^6 \pm 0.09$	$2.1 \times 10^5 \pm 0.16$
	Muscle	$0.2 \times 10^2 \pm 0.03$	nd	nd
II	Kidney	$2.6 \times 10^7 \pm 0.08$	$3 \times 10^6 \pm 0.11$	$3.4 \times 10^5 \pm 0.15$
	Liver	$1.8 \times 10^7 \pm 0.07$	$2.2 \times 10^6 \pm 0.08$	$1.8 \times 10^5 \pm 0.09$
	Muscle	$1.3 \times 10^2 \pm 0.12$	$0.1 \times 10^2 \pm 0.05$	nd
III	Kidney	$2.6 \times 10^7 \pm 0.12$	$2.5 \times 10^6 \pm 0.1$	$3.9 \times 10^5 \pm 0.14$
	Liver	$2.1 \times 10^7 \pm 0.09$	$2 \times 10^6 \pm 0.07$	$2.1 \times 10^5 \pm 0.18$
	Muscle	nd	nd	nd
IV	Kidney	$1.2 \times 10^7 \pm 0.11$	$1.7 \times 10^6 \pm 0.14$	$3.8 \times 10^5 \pm 0.14$
	Liver	$1.6 \times 10^7 \pm 0.08$	$1.4 \times 10^6 \pm 0.08$	$4.2 \times 10^5 \pm 0.12$
	Muscle	$0.8 \times 10^2 \pm 0.06$	nd	nd
V	Kidney	$3.5 \times 10^7 \pm 0.1$	$3.3 \times 10^6 \pm 0.11$	$4.3 \times 10^5 \pm 0.12$
	Liver	$2.2 \times 10^7 \pm 0.09$	$2.2 \times 10^6 \pm 0.12$	$2.2 \times 10^5 \pm 0.09$
	Muscle	nd	nd	nd

Group of control fish*	Part(s)	Copy number 16S rDNA gr^{-1} tissue ± SD***	Copy number toxR gr^{-1} tissue ± SD***	Colony-Forming Units (CFU) gr^{-1} tissue ± SD**
I	Kidney	nd	nd	nd
	Liver	nd	nd	nd
	Muscle	nd	nd	nd
II	Kidney	nd	nd	nd
	Liver	nd	nd	nd
	Muscle	nd	nd	nd

ND, No amplification detected.
* Each group was composed of 10 specimens.
** *V. anguillarum* levels were determined by plate counts on tryptic soy agar spread plates.
*** Real-time PCR determination of *V. anguillarum* concentrations was based on the mean of triplicate samples. Concentrations were derived from a standard curve using the mean of triplicate Ct values for serial tenfold dilutions of DNA extracted from known concentrations of bacteria.

5.4 Discussion

Vibrio anguillarum causes a high incidence of disease in continental and marine aquaculture. One of the most affected by the infection is the sea bass (*Dicentrarchus labrax)*.

The specific identification of *Vibrio anguillarum* and the relative cells number in the host tissue is an useful information to understand the dynamic of infection. Previously, many studies have been carried out on methods and targets for the detection of *V. anguillarum* in fish tissue (Grisez *et al.* 1997; Blanch *et al.* 1997; Chair *et al.* 1994; Nadkarni *et al.* 2002) but, due to the high diversity of microbial community inhabiting fish tissue, it is difficult to identify *V. anguillarum,* specially by the use of culture-dependent techniques (Cahill, 1990; Spanggaard *et al.* 2001; Anderson, 1995). On the other hand, molecular methods require an accurate choice of target and probes for unambiguous detection of the object.

The present study is aimed to the assessment of two genes (*16SrDNA* and *toxR*) as targets for the detection of *V. anguillarum* in *D. labrax* tissues by means of real time PCR. Moreover was evaluated the influence of the different operon copy number on the sensitivity of the detection.

toxR is a chromosomally encoded gene whose principal function is the regulation of the expression of several virulence factors (Li *et al.* 2000; Provenzano *et al.* 2001; Krukonis *et al*. 2000). Moreover, the expression of *toxR* gene seems to enhance bile resistance and other environmental stimuli (Provenzano *et al.* 2000; Peterson, 2002) Previous studies developed genetic identification methods based on the use of *toxR* both for isolated strains or directly from marine environment (Kim *et al.* 1999; Martinez-Picado *et al.* 1996). In addition, although the *toxR* seems to be widely

distributed in the family of Vibrionaceae (Vuddhakul *et al.* 2000), the interspecies homology values are much lower than those of *rRNA* genes (Osorio and Klose, 2000). Indeed Okuda *et al.* (2001) confirmed that, both the DNA probe and PCR methods established, specifically detected the *toxR* gene sequence in *V. anguillarum* but not in other species of the genus Vibrio. Although the number of *toxR* operon in *Vibrio anguillarum* is not still known, other strains belonging to the same genus contain a single operon coding for *tox* homologous (DiRita and Mekalanos, 1991; Miller *et al*. 1989; Lin *et al.* 1993; Fe Franco and Hedreyda, 2006). In our case, the results of the quantification in pure culture show that the number of copies of *toxR* detected for each sample matches the amount of the cells specially added after photometer detection and calculation of copies number with Whelan equation, both for each standard point and samples collected during the growth phases. Due to the coincidence of values n°cell = n°*toxR* copies we assumed that one cell of *V. anguillarum* contains one copy of *toxR* and we considered it as a reference for the next quantitative measurements.

Although *16SrDNA* is considered as a suitable marker gene for taxonomic and phylogenetic applications, there are many different concepts regard the rrn operon number in quantitative *16SrDNA*-based experimental systems. Several authors suggest that the rrn copy number is a negligible factor in quantification of bacteria from a mixed culture (Fogel *et al.* 1999).

While other studies (Nadkarni *et al.* 2002; von Wintzingerode *et al.* 2000) reveal that PCR is influenced by variation in the number of operons, which is related the metabolic status and the generation time of the bacteria at the time of sampling (Farrelly *et al.* 1995).

Among the bacterial species, the *16SrDNA* copy number varies considerably from 1 to 15 (Klappenbach *et al.* 2000; Charles and Ishikawa, 1999). The same range was evaluated specially for Vibrio sp. (Reen *et al.* 2006). The number of operons of *16SrDNA* of the strain 975/I is unknown. However in all the assays carried out in this study (standard curve dilutions, growth phases and fish tissue) the ratio between *16SrDNA* and *toxR* genes copy number detected averaged around 9.40 which let hypothesize the presence of about 9 rrn copies number of *16SrDNA* respect of the single copy of *toxR* gene. The use of real time PCR as a method for the detection of genes operons number is currently used (Kim and Wang, 2009; Lee *et al* 2008) and for our knowledge, is the first indication about *16SrDNA* copy number in *V. anguillarum* strains.

Concerning the specificity on the detection, both targets provided similar results, thus taking into account the normalization of data we can use both for the identification and quantitative detection of *Vibrio anguillarum*. In fact, although primers designed for both genes provided a specific amplification equally in pure culture and in fish tissue, a direct reading can be obtain using *toxR* as a target gene, while *16SrDNA* could be a good target to increase the sensitivity of detection.

The simultaneous use of both targets could be an interesting application to obtain a cross-check in case of application in fish other than *Dicentrhancus labrax* which could host different microbial community. The use of taxonomic and functional genes offers the vantage of a phylogenetic identification together with information about pathogenic potential of detected microorganisms.

The sensitivity of the described method ranged around 2×10^3 cells g^{-1} fish tissue obtaining results in 3 to 4 h compared to 48 h from conventional plate counts

normally incubated at 20 °C. The bacterial load determined in tissue is not greatly distant from data from that previously reported in the literature (Rehnstam *et al*. 1989).

In conclusion, the method proposed in this study could be a useful tool for the sensitive and accurate detection of *Vibrio anguillarum* in fish tissue.

5.5 Reference

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Summarizing Conclusions

Summarizing conclusion

I. Environmental stress induces the expression of virulence genes in *Vibrio anguillarum*. The response to stress conditions is different in the two strains tested: *Vibrio anguillarum* 975/I serovar O1, virulent strain, carrying the plasmid pJM1 responds to environmental stimuli with the expression of genes involved in infection process, while *Vibrio anguillarum* ATCC43307 serovar O3, a-virulent strain, plasmidless enhanced the expression of *toxR* and *fur* which regulate phenotypic modification in relation to environmental changes in several pathogens.

II. Temperature enhances *empA* expression in *Vibrio anguillarum*; comparing EmpA production in different media, it is noticeable the influence of nutrients and salinity (NaCl %) in the post-transcription control of EmpA;

III. Real Time PCR is a rapid and specific method for the determination of *Vibrio anguillaum* in infected fish; *16SrDNA* and *toxR* can be target genes for the detection of *Vibrio anguillarum* in fish tissues.

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