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Impact of different parameters upon the expression of certain virulence factors of nonhalophilic and halophilic *Vibrio* strains

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ANCA-MICHAELA ISRAIL, CRISTINA DELCARU, MARIANA –CARMEN BALOTESCU CHIFIRIUC
National Institute for Research and Development in Microbiology and Immunology Cantacuzino, Spl. Independentei 103, 060031 Bucharest, Romania
E-mail: ancaisrail@yahoo.com

Abstract

The purpose of the present study was to investigate the expression of 13 virulence factors (VF) in nonhalophilic and halophilic vibrios isolated from clinical cases and external medium, in different cultivation conditions simulating environmental stress factors.

Some of these strains displayed positive slime and indole reaction at 4 ^{0}C, demonstrating the bacterial ability to survive in stress conditions by biofilm development and preservation of certain active enzymes. Haemolysins, lecithinase and aesculinase were better expressed at 28^{0}C than at 37^{0}C, demonstrating the dual role of these enzymes in microbial surviving and adaptation to external medium as well as to homeotermic hosts.

The ability of surviving and synthesis of different VF was present both in non-halophilic and halophlic strains in the presence of certain limits of NaCl concentrations. Kanagawa toxin was present not only in V. parahaemolyticus but also in V. metschnikovii, V. alginolyticus, V. vulnificus, V. fischeri and V. anguillarum.

The halophilic vibrios proved higher resistance to broad range of pH (1.0-9.2), than non-halophilic strains, the most resistant proving to be V. vulnificus, followed by V. fischeri and V. anguillarum, expressing 4 VF (mucinase, lecithinase, gelatinase, aesculinase), whereas non-halophilic vibrios cultivated starting only from pH 6.0-6.5 (7 active enzymes), reaching the highest number of 12 VF at pH.7.2 decreasing thereafter to pH 9.2 at 7 VF.

The different glucose concentrations (1.5-3.0%) in the culture media proved to have more evident and variable effects on halophilic than upon non-halophilic strains, with an evident increasing effect especially on V. vulnificus sp. on one side, as well as on caseinase expression in different species, on the other side.

In conclusion, the present results demonstrated the higher resistance of halophilic vibrios in different environmental stress conditions as well as their particular high virulence and resistance to acid pH, during the gastric infection in humans (similar to Campylobacter pylori).

Keywords: halophilic, enzymes, quorum-sensing, virulence/mucinase.

Introduction

In the last years, the scientists have become more and more interested in the study of the influence of different environmental parameters (3, 12) upon the estuarine and coastal ecosystems (36) in the purpose to establish the relationship between these aspects and the human health in the new conditions required for the sanitation security concerning the recreation time and modern tourism industry (4, 5, 11, 16).

It must be pointed out that the most important marine coastal bacteria are the vibrios characterized by the double life-style i.e. marine and animal (human and mammals) enteric life (1, 6, 8, 17, 18).

Vibrio species are surviving in the aquatic environment in complex multispecies

biofilm structures attached on various biotic (chitinous exoskeleton of zooplankton) and abiotic surfaces, the biofilm promoting interspecies (7, 18, 31) metabolic and genetic collaboration as well as protection against different environmental stress conditions (27, 28), affecting at the same time the spreading of the respective vibriosis in human and animal population.

Many *Vibrio* species (*V. cholerae, V. vulnificus, V. anguillarum*) have developed also long term starvation strategies (viable dormant and non-cultivable state), that allow them to survive for months and even years in water and sediments.

At the same time, many vibrios synthesizing enzymes such as chitinases, active on aquatic substrate as well as other 10 enzymes with chitinase-like activity (8) and iron-chelating systems, that are releasing metabolic products used as source of energy contributing in this way to survival in the environment, although their roles are still to be studied.

In the last years, in the developed countries, it was evidenced an increasing number of infections with non-cholerigenic vibrios as *V. cholerae* non O1/nonO139, as well as with *V. parahaemolyticus, V. vulnificus* (50% - 60% fatal cases) and *V. fluvialis* (as cholera-like cases of entorocolitis in adults 64% and children 36%) (2, 13).

This aspect has become more an more stringent when considering that approximately 60% of the world population is living in the first 150 km distance from the marine coast and a new increase of the population in these areas is being prospected for the next decades. At the same time the estuarine and coastal areas are submitted to permanent anthropic inputs with role of stress factors able to cause new changes in the ecosystem conditions as eutrophisation and decline of water quality (4, 28, 29, 30).

The natural (storms, floods, mud nutrients) (12, 19, 22, 27) and anthropic (the way of land using, the increasing of human input) changes have proved of having strongly influenced the biology of marine bacteria, in some areas of the world certain marine bacteria even being considered new emergent pathogens due to the recent great number of reported cases (i.e. pandemic foodborne gastroenteritis after consumption of seafood contaminated by *V. parahaemolyticus* new serotype O3 K6, described in China, Japan, Korea, Vladivostok up to the coast of Great Britain, France, Spain, Adriatic and Black Sea until now) (9,10, 21, 37). The explanation of the emergence of new pathogens from opportunistic bacteria remains still not completely understood.

Some investigators admitted that these changes in the biology of vibrios could be related to the climate changes, as high temperature (22, 24, 25, 26, 34, 38), hurricanes (12), rainfalls, floods salinity and other osmotic variations, pollution with organic nutrients (N, P level), association with other marine organisms (phyto- and zoo-plankton), occurrence of new degrees of susceptibility of certain fish species to certain new serogroups of vibrios (e.g. *V. parahaemolyticus, V. anguillarum*), increasing of sea food consuming (35), mode of seafood preservation and at least but not the last, the increasing number of immunocompromised human-organisms. But the most important factor responsible for outbreak of vibriosis remains the temperature, these outbreaks being not detected in water with a temperature below 14-16^{o}C. In the present study, the authors have tried to investigate the influence of some parameters as temperature, salinity, pH, glucose concentrations upon the expression of 13 virulence factors of nonhalophilic and halophilic vibrios isolated from the local ecosystems of Danube Delta and marine coast of the Black Sea.

Materials and Methods

Bacterial strains

Originating in the strains collection of *Vibrio* Laboratory – National Institute for

Research and Development in Microbiology and Immunology Cantacuzino:

- 30 nonhalophilic *Vibrio* strains, out of which:
  - 19 strains of *V. cholerae* O1
    - 12 clinical cases (outbreaks 1990 - 1994)
    - 7 water sources (1990 - 1994)
  - 11 strains of *V. cholerae* non O1
    - 5 clinical isolated cases
    - 6 water sources
- 28 halophilic *Vibrio* strains isolated from different water sources: 5 strains of *V. metschnikovii*, 5 *V. parahaemolyticus*, 5 *V. alginolyticus,* 3 *V. vulnificus,* 5 *V. fischeri* and *5 V. anguillarum*;
- 1 strain of *V. cholerae O1 classical biotype serotype Inaba* 35A-USA (used as positive control for oxidase reaction);
- 1 strain of *V. cholerae O1 classical biotype serotype Ogawa* NIH 41 USA (as positive control for oxidase reaction);
- 1 strain of *Escherichia coli ATCC 25922* (as negative control for oxidase reaction);
- 1 strain of *Salmonella typhimurium NCTC 3717* (as negative control for oxidase reaction);
- 1 strain of *Salmonella enteritidis gärtner* (as negative control for oxidase reaction).

Culture media

There were used 13 media (already described by the authors) (20) to identify the presence of 13 soluble VF, i.e. the production of pore-forming-toxins (sheep red blood cells haemolysis - SRBCH), CAMP-like factor, Kanagawa enterotoxin, lipase, lecithinase), proteases (caseinase, gelatinase), glucidases (amylase, mucinase, aesculin hydrolysis), DN-ase, triptophanase production as well as the adherence ability to abiotic surfaces quantified by slime test.

Methods

Strains identification

Biochemical identification was carried out by 36 conventional tests: TSI (triple sugar agar), oxidase, nitrate reduction, motility, growth at 37°C, D-glucose fermentation (with/without gas production assessed by Durham tube), growth at different NaCl concentrations (0.5%, 1.0%, 3.0%, 6.0%, 8.0%, 10.0%) (32), ornithine-decarboxylase, lysine-decarboxylase, arginin-dihydrolase, lactose, sucrose, manitol, mannose, maltose, arabinose, dulcitol, adonitol, inositol, sorbitol, galactose, Na citrate Simmons, indole production, methyl red and Voges Proskauer (on Clark medium) reactions, phenylalanine-desaminase, sensitivity to O/129 vibriostatic agent (2,4,-diamino-6.7-diisopropylopteridine) 400 µg/disk (prepared in house), ONPG (ortonitroparagalactoside) (39).

Determination of the virulence factors (VF) was done following the corresponding methods already described (20)

***Vibrio* strains resistance and expression of VF at different incubation temperatures**:

The bacterial growth rate and curves at different temperatures were established by measuring the absorbance of bacterial cultures at 600 nm at different intervals (0, 2, 4, 6, 12, 24, 48 and 72 hrs). The 58 studied strains were spotted simultaneously on the special media for virulence factors in three identical Petri dishes, the first one being incubated at 4°C, the second at 28°C and the third one at 37°C.

Vibrio strains resistance and expression of VF at different degrees of salinity:

Only 38 (1o non-halophilic *V. cholerae* and 28 halophilic) *Vibrio* strains were spotted simultaneously on the special media (for virulence factors) prepared with different NaCl concentrations, i.e.: 0.5%, 1.0%, 3.0%, 6.0%, 8.0%, 10.0%.

Dependence of growth and expression of VF in nonhalophilic and halophilic *Vibrio* strains on the pH of the culture media.

The same 38 *Vibrio* strains were tested in two different methods:

a) the strains were spotted on special solid media for VF prepared with different pH from 1.0- 9.2 and thereafter incubated 24 hrs at 37 ^{0}C

b) the strains were inoculated and preserved 24 hrs at 37 ^{0}C in different dilutions of acetic acid solution (pH 1.0-9,2) and thereafter spotted on special solid media for VF with pH 7.2.

Dependence of growth and expression of VF in non-halophilic and halophilic vibrio upon the glucose concentration of the culture media:

The same 38 *Vibrio* strains were spotted on special solid media for VF, containing 0%, 1.5% and 3.0% glucose concentrations and thereafter incubated 24 hrs at 37^{o}C.

Results

Out of 13 virulence tests, the slime (adherence to abiotic surface) and indole production were the only positive at 4^{0}C temperature of incubation in non-halophilic (4/30 and respectively 9/30) and halophilic (5/28 and respectively 4/28) vibrios, demonstrating the resistance of these strains in thermal stress conditions (table no. 1).

Table no. 1. Influence of the incubation temperature upon the expression of virulence factors in non-halophlic and halophilic *Vibrio* strains

***Vibrio* species**	**No. strains**	**Adhesion to abiotic substrate**			**Pore-forming toxins**										**Proteases**							**Glucidases**						**DN-ase**	
		*			CAMP factor		SRBCH		Kanagawa Haem-olysin		Lipase		Lecithinase		Caseinase		Gelatinase		Tryptophanase			Mucinase		Amylase		Aesculinase			
		4	28	37	28	37	28	37	28	37	28	37	28	37	28	37	28	37	4	28	37	28	37	28	37	28	37	28	37
Non halophilic strains																													
Vibrio cholerae O1 Acute diarrhea	12	0	10	7	2	2	6	4	0	4	1	2	9	10	2	5	6	12	4	5	8	4	4	10	12	1	1	5	5
Waters	7	1	6	6	2	1	3	5	1	4	0	1	4	5	1	3	3	7	2	4	1	1	1	3	5	2	3	4	5
Vibrio cholerae non-O1 Acute diarrhea	5	0	1	3	0	0	3	3	3	3	0	0	3	3	1	0	0	3	0	2	2	0	0	1	2	0	2	2	5
Waters	6	3	3	6	2	1	6	6	2	4	0	0	5	5	4	4	6	6	3	2	4	5	5	6	6	0	0	5	5
Total no. of non-halophilic strains	**30**	**4**	**20**	**22**	**6**	**4**	**18**	**18**	**6**	**15**	**1**	**3**	**21**	**23**	**8**	**12**	**15**	**28**	**9**	**13**	**15**	**10**	**10**	**20**	**25**	**3**	**6**	**16**	**20**
Halophilic strains																													
Vibrio metschnikovii	5	1	4	3	0	0	0	2	2	2	0	1	2	2	2	1	2	4	1	3	o	0	4	2	2	3	4	3	4
V. parahaemolyticus	5	0	0	5	0	0	0	2	0	4	1	1	3	2	0	2	1	2	0	2	1	0	2	0	3	2	0	0	0
V. alginolyticus	5	0	0	1	0	o	0	0	0	1	0	1	0	5	0	o	0	0	0	2	0	3	5	0	1	0	0	0	0
V. vulnificus	3	1	0	1	0	2	1	2	2	0	1	2	2	o	0	1	2	3	0	3	o	0	1	0	1	3	3	1	1
V. fischerii	5	1	2	1	2	2	0	1	0	2	3	5	3	2	1	0	3	5	0	2	1	0	2	0	1	5	5	1	2
V. anguillarum	5	2	3	5	1	4	3	4	0	1	0	4	0	5	2	4	0	5	3	4	o	3	3	0	2	5	5	1	1
Total no. of halophilic strains	**28**	**5**	**9**	**16**	**3**	**8**	**4**	**11**	**4**	**1o**	**5**	**14**	**10**	**16**	**5**	**8**	**8**	**19**	**4**	**16**	**2**	**6**	**17**	**2**	**10**	**18**	**17**	**6**	**8**

*Incubation temperature

Even though certain VF were better expressed at 28^0C than at 37^0C (i.e. haemolysins CAMP-like factor, indole and slime in *V. cholerae*, lecithinase, tryptophanase and Kanagawa toxin in *V. vulnificus*, tryptophanase in *V. fischeri*, caseinase and slime in *V. metschnikovii* and tryptophanase in *V. anguillarum*), however the highest expression of VF in *Vibrio* strains was noticed at 37^0C, this aspect demonstrating that the pathogenic potential of some strains can be expressed only in homeothermic organisms, whereas at 28^oC (in conditions similar to the external medium) this potential remains unexpressed (or suppressed) (table no. 1).

The *V.cholerae* O1 and non O1 strains isolated in clinical cases expressed high level of lecithinase, whereas all *V. cholerae* O1 and non O1 strains, irrespective of their source of isolation, expressed high positivity for gelatinase, amylase DN-ase and slime test (table no. 1).

Kanagawa test revealed the presence of pore-forming Kanagawa-toxin, not only in *V. parahaemolyticus* strains, but also in other halophilic (*V. alginolyticus, V. vulnificus, V. fischeri, V. anguillarum*) (Fig. 3) and non-halophilic vibrios (*V. cholerae* non O1) (table no. 1 and Fig. 1).

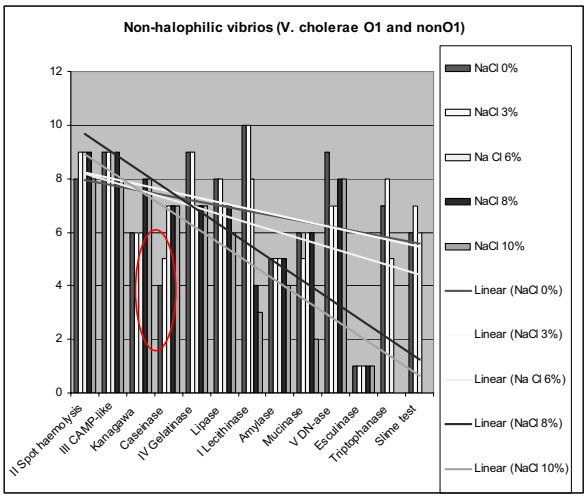

Fig. 1. Levels of VF related to different NaCl concentrations in *V. cholerae* strains

In *V. cholerae* O1 and non O1 strains, the expression of VF has gradually increased from NaCl concentration of 0.5% to 6.0%, followed by an evident decreasing at 8.0% and 10% (Fig. 1). The expression of certain enzymes was correlated to different NaCl concentrations, i.e. lecithinase, lipase, gelatinase were expressed predominantly at low concentration of 0.5-3.0 % whereas pore forming toxins (haemolysins, Kanagawa toxin and CAMP-like factor) and caseinase were better expressed at higher NaCl concentrations (8%).

In *V. metschnikovii* strains, the level of VF expression was lower than that of *V. cholerae* O1 and non *O*1 strains. The highest levels were expressed by mucinase, gelatinase, DN-ase, lecithinase and esculinase at 0.5-3.0%, followed by adhesion ability to abiotic surface at 0.5-6.0% and caseinase at 6.0-8.0% NaCl concentrations (see Fig. 2 where the Roman number are indicating the frequency of VF expression).

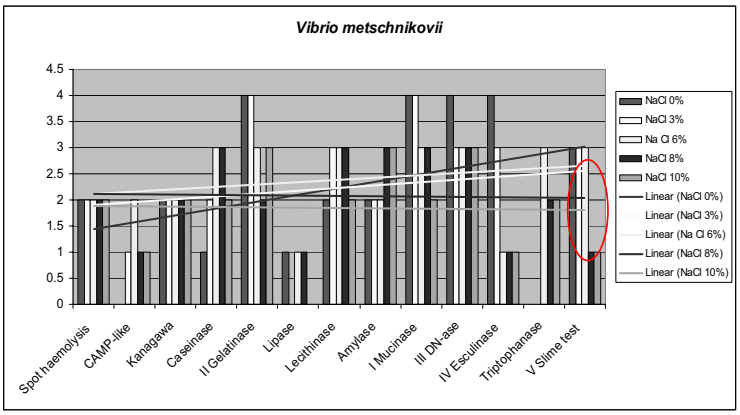

Fig. 2. Levels of VF related to different NaCl concentrations in *V. metchinikovii* strains

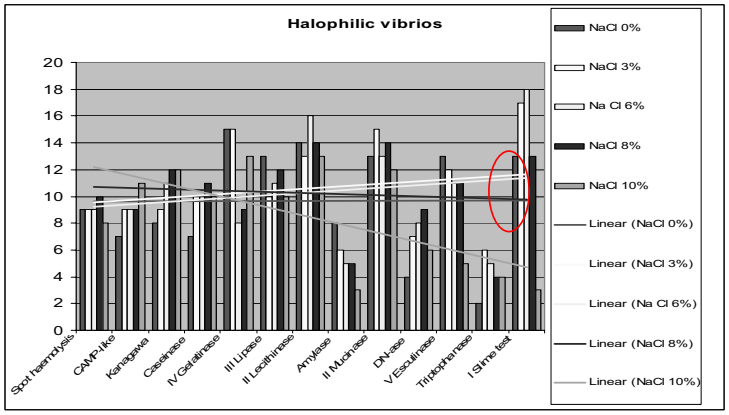

Fig. 3. Levels of VF related to different NaCl concentrations in halophilic vibrios

In *V. parahaemolyticus* the highest levels of positivity were registered for adhesion ability to abiotic substrate (0.5-6.0%), mucinase (3.0-8.0%) and lecithinase (8-10%) NaCl concentrations (Fig. 4).

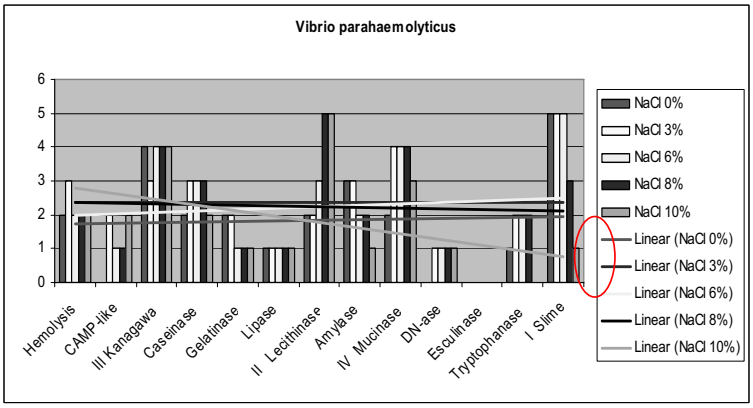

Fig. 4. Levels of VF related to different NaCl concentrations in *V. parahaemolyticus* strains

High levels for Kanagawa haemolysins were constantly expressed by all NaCl concentrations.

In *V. alginolyticus*, the highest levels were registered for mucinase followed by lecithinase, at all NaCl concentrations, aspect suggesting that in halophilic vibrios, the enzymatic activity is less influenced by this parameter (Fig. 5).

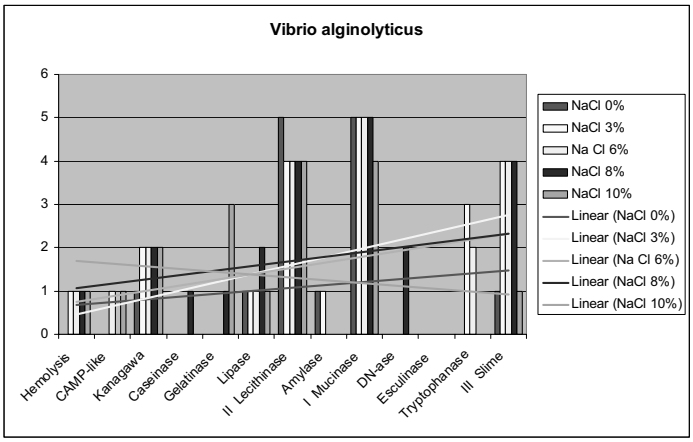

Fig. 5. Levels of VF related to different NaCl concentrations in *V. alginolyticus* strains

The adhesion ability to abiotic surface was preferentially expressed at 3.0-8.0 % NaCl concentration.

In *V. vulnificus*, the spectrum of VF was very narrow, these strains expressing high levels of gelatinase (0.5-3.0%), aesculinase and mucinase (expressed preferentially at 0-8.0% NaCl concentrations) (Fig. 6).

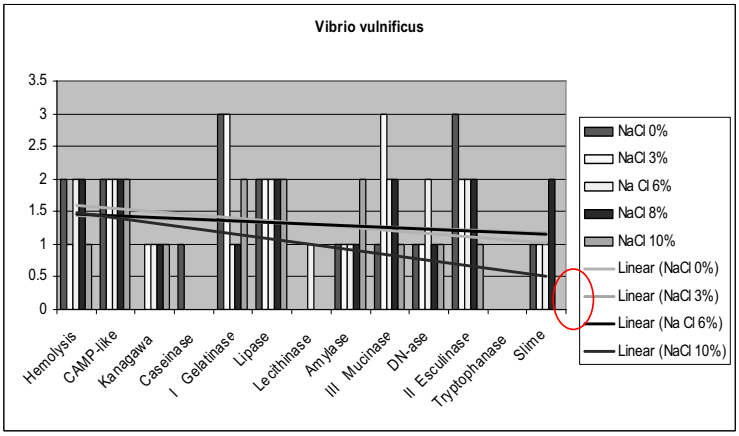

Fig. 6. Levels of VF related to different NaCl concentrations in *V. vulnificus* strains

The *V. fischeri* strains displayed the highest levels by esculinase (0.5-10%), gelatinase (0-10.0 %), lipase (0.5-10.0%) and adherence to abiotic surfaces (3-8.0%) (Fig. 7).

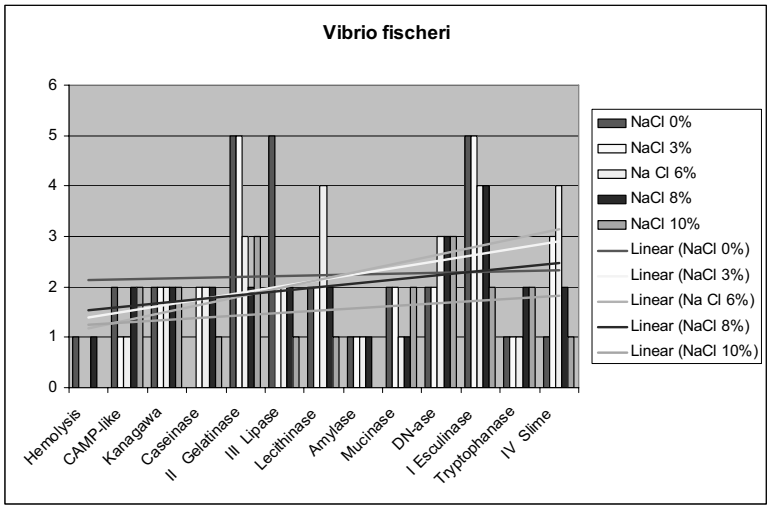

Fig. 7. Levels of VF related to different NaCl concentrations in *V. fischeri*

V. anguillarum, by comparison with all other halophilic vibrios, exhibited the highest number and expression levels of VF, the most of them expressed in large limits of NaCl concentrations, i.e.: gelatinase (0.5-3.0%), lecithinase, slime (0.5-6.0%), aesculinase (0.5-8.0%), caseinase, lipase, haemolysins and CAMP-like factor (0.5-10%) (Fig. 8).

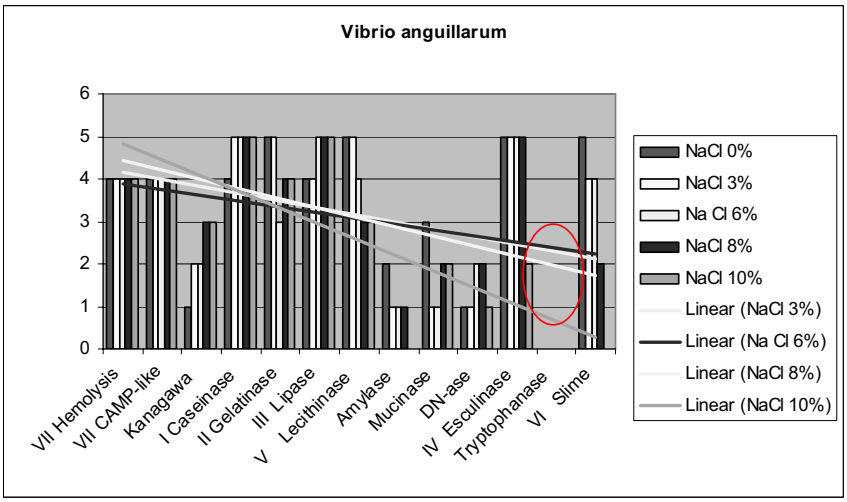

Fig. 8. Levels of VF related to different NaCl concentrations in *V. anguillarum*

Our results demonstrated high levels of mucinase expression in broad limits of salinity in different non-halophilic and halophilic vibrios, i.e.: *V. cholerae* O1 and nonO1, *V. metchinikovii, V. anguillarum* (0.5-8.0%), *V. parahamolyticus* (3.0-8.0%), *V. alginolyticus* (0.5- 10.0%), *V. vulnificus* (3.0%).

In many cases, it could be noticed the constant simultaneous expression of three virulence factors, i.e. mucinase, lecithinase and lipase in *V. cholerae* O1 and nonO1, *V. metchinikovii, V. alginolyticus, V. anguillarum*.

As far as the dependence of growth and VF virulence expression in *Vibrio* strains on

the pH of the cultivation media is concerned, when the microbial strains were spotted on special solid media for VF and with pH large limits between 1.0-9.2, after 24 hrs incubation at 37^0C, *V. vulnificus* proved to be the most resistant, expressing 4 VF (mucinase, lecithinase, gelatinase and esculinase) even on media with pH 3.5.-5.0 (Table no. 2, method a).

Table no. 2. Influence of the pH of the culture medium upon the expression of virulence factors in non-halophilic and halophilic *Vibrio* strains

Bacterial strain	pH 1- 3	pH 3,5-5	pH 5,5	pH 6-6,5	pH 7,2	pH 9,2
	Virulence Factors (b)	Virulence factors (a)	Virulence factors (b)	Virulence factors (b)	Virulence factors (b)	Virulence factors (b)
Vibrio cholerae O1 and non O1 NG/7/12/7	NO GROWTH			SRBCH Kanagawa Gelatinase Caseinase Lecithinase DN-ase Mucinase	CAMP, SRBCH Kanagawa Lipase, Amylase Gelatinase, Caseinase Lecithinase, DN-ase Mucinase Tryptophanase ,Slime test	SRBCH Kanagawa Lipase Amylase Gelatinase Lecithinase Mucinase
V. metschnikovii 1/4/7/5			Mucinase	Mucinase, Gelatinase Lecithinase Aesculinase	Mucinase Gelatinase, Lecithinase Aesculinase, DN-ase Tryptophanase, Slime test	Mucinase Gelatinase Lecithinase Aesculinase Slime test
V. parahaemolyticus 3/6/6/4			CAMP Mucinase Slime test	CAMP Mucinase, Lipase Lecithinase DN-ase, Slime test	Mucinase 22 ^{0} C Lipase, Lecithinase Gelatinase, Aesculinase Slime tests	Mucinase 22 ^{0} C Lipase Lecithinase Gelatinase
V. alginolyticus 5/6/10/5			CAMP, Kanagawa Mucinase 37 ^{0} C Lipase, Slime test	CAMP., Kanagawa Mucinase 22 ^{0}C and 37 ^{0} C Lipase, Gelatinase Slime test	CAMP Kanagawa, Mucinase Lipase, Amylase Gelatinase, Caseinase Lecithinase, DN-ase Slime test	Kanagawa Mucinase Amylase ++ Gelatinase Slime test
V. fischeri 4/5/5/4	Mucinase Lecitinase Gelatinase Aesculinase		Gelatinase Lecithinase Mucinase Aesculinase	CAMP, Gelatinase Lecithinase,Mucinase Aesculinase	Gelatinase Mucinase Lecithinase 22 ^{0} C Caseinasee Aesculinase	Gelatinase Lecithinase Mucinase Aesculinase
V. anguillarum 7/9/10/7	Mucinase Lecitinase Gelatinase Aesculinase		SRBCH, Kanagawa Lecithinase Gelatinase Mucinase Aesculinase Slime test	CAMP, SRBCH Kanagawa Gelatinase, Mucinase Lecithinase, DN-ase Aesculinase Slime test	CAMP, SRBCH Kanagawa, Gelatinase Mucinase, Caseinase Lecithinase, DN-ase Aesculinase Tryptophanase	SBCH +/- Gelatinase Lecithinase Mucinase DN-ase Aesculinase Slime test.
V. vulnificus 4/8/8/11/6	Mucinase Lecitinase Gelatinase Aesculinase	Mucinase Lecitinase Gelatinase Esculinase	CAMP, SRBCH Kanagawa Mucinase, Gelatinase Caseinase, DN-ase Aesculinase	CAMP, SRBCH Kanagawa, Mucinase Gelatinase Caseinase, DN-ase Aesculinase	CAMP, SRBCH Kanagawa, Mucinase Gelatinase, Caseinase Lecithinase, DN-ase Aesculinase Tryptophanase, Lipase	SRBCH Mucinase Gelatinase Caseinase DN-ase Asculinase

Legend:

- **white** - Microbial strains spotted on solid media for virulence factors (VF) with different pH (1.0-9.2): *V. vulnificus* proved to be the most resistant exhibiting 4 VF at pH 3.5;
- **light grey -** Microbial strains preserved 24 hrs in solutions of acetic acid with different pH (1.0-9.2) and then transferred on solid media for VF (pH 7.2): the most resistant proved to be 3 halophilic vibrios: *V. vulnificus, V. fischeri* and *V. anguillarum* preserved even at pH 1.0-3.5, expressing 4 VF (lecithinase, mucinase, gelatinase, aesculinase)
- **dark grey** – No growth

When microbial strains preserved in advance 24 hrs in different dilutions of acetic acid solution (with different pH from form 1.0- 9.2) and thereafter transferred on solid special media for VF, after 24 hrs incubation at 37°C, the most resistant proved to be 3 halophilic species: *V.vulnificus, V. fischeri* and *V. anguillarum* expressing even after being preserved 24 hrs in pH 1.0-3.5, 4 VF (lecitinase, mucinase, gelatinase, aesculinase) (Table no. 2, method b).

In both experiments, the other 3 halophilic *Vibrio* species, cultivated and started to express VF, only at pH 5.5 (Table no. 2), the number of expressed VF increasing up to pH 7.2 and decreasing thereafter up to 9.2.

In case of nonhalophilic vibrios, *V. cholerae* O1 and non O1 strains, treated in the same above-mentioned conditions, cultivated and expressed a number of 7 VF only at pH 6.0-6.5, the number of positive VF increasing to 12 at pH 7.2 and decreasing thereafter to 7 at pH 9.2 (Table no. 2).

The presence of different glucose concentration (1.5% and 3.0%) in the culture media proved to have different effects on the expression of VF (Table no. 3) (either increasing, decreasing up to total inhibition of VF or no effect at all). The highest positive effect of both glucose concentrations was observed on one side, for *V. vulnificus* strains, consisting of an increased expression of CAMP-like factors, Kanagawa toxin, lipase, caseinase, DN-ase and on the other side, the expression of caseinase in *V. cholerae* strains and 4 (*V. alginolyticus, V. vulnificus, V. fischeri* and *V . anguillarum*) of the 6 halophilic species.

Table no. 3. Influence of the glucose concentration upon the expression of virulence factors in non-halophilic and halophilic *Vibrio* strains

	[Glc]	CAMP	SRBCH	Kanagawa	Lipase	Amylase	Gelatinase	Caseinase	Lecithinase	DN-ase	Mucinase	Esculinase	Tryptophanase	Slime
V. cholerae	1,5 %													
	3.0%													
V. metschnikovii	1,5 %													
	3.0%													
V. parahaemolyticus	1,5 %													
	3.0%													
V. alginolyticus	1,5 %													
	3.0%													
V. vulnificus	1.5 %													
	3.0%													
V. fischeri	1.5 %													
	3.0%													
V. anguillarum	1.5 %													
	3.0%													

Legend: **white** - no change; **black** – disappearance or decreasing effect; **grey** – increasing effect

Discussion

It is well known today that *Vibrio* species are located in biofilm structures developed on / in different marine organisms or on abiotic surfaces. Biofilms (18) contribute to surviving of bacterial communities, favoring the metabolic and genetic correlation among the bacterial

species as well as the common protection against the environmental stress conditions playing at the same time an important role in the evolution of human infections (outbreaks).

The pathogenicity of vibrios has proved to be temperature dependent, the respective infections being observed especially in areas with relatively high sea temperatures and related to global warming or other environmental conditions favoring the bacterial infectivity. A recent study of 25 outbreaks of sea fish diseases proved to be caused by bacterial agents of which 65% of isolated strains belonged to genus *Vibrio*.

The simultaneous positivity of slime and indole reaction at 4^{o}C in certain nonhalophilic and halophilic *Vibrio* strains revealed by our studies, come into accordance with Bartlett's (6) and Kovacicova's et al. (23) mentioning that a close interrelation could be established between these two reactions, determined by the presence of certain genes with double regulation mechanisms for acetoin biosynthesis (indole production) and mobility/biofilm formation (slime test in *Vibrio* strains).

The aspect that in certain *V. cholerae* strains, some VF were better expressed at 28^{o}C than at 37^{o}C, when correlated with lower bacterial growth rates determined by our testings, is pleading for the hypothesis that these virulence factors (haemolysins, CAMP-like factor, triptophanase reaction, slime factor) are positively regulated in conditions of lower bacterial densities, corresponding to a lower level of autoinducers responsible for QS phenomenon and expression of virulence factors in bacteria (7, 23). The high adherence ability of *V. cholerae* strains to abiotic surfaces could explain their high resistance in the external environment and their high incidence of isolation during interepidemic periods (20).

Whereas cultivated on simple broth supplemented with different NaCl concentrations, the non-halophilic and halophilic vibrios proved to fit in the standard limits of NaCl concentration, when cultivated on special media for VF, the respective strains exhibited growth in a broad range of NaCl concentrations, demonstrating their higher resistance to osmotic stress in conditions of cultivation on complex culture media.

In *V. cholerae* O1 and non O1 strains, the expression of VF has gradually increased from 0.5% to 6.0%, followed by an evident decreasing at 8.0 and 10% (Table no. 1). In *V. metschnikovii,* the pattern of VF expression was approximately similar to that observed in *V. cholerae* strains, although the individual levels of VF expression was much lower (Fig. 2).

The constant high adhesion ability of *V. metschnikovii*, to the abiotic surface at 0.5-6.0% NaCl is pleading for the hypothesis that colonization with *V. metschnikovii* is probably promoted in waters with the respective levels of salinity.

By comparison, in halophilic *Vibrio* strains the expression of VF was approximately constant between 0.5 to 8.0% with a sudden decrease at 10.0% NaCl concentration (Fig. 3).

In *V. anguillarum* strains, the high frequency of a great number of VF (surface colonization ability, lecithinase, haemolysins, aesculin hydrolysis) expressed in very large limits of NaCl (0.5 – 10.0%) are pleading for the high pathogenic role of these vibrios responsible for persistent endemic outbreaks in fish aquacultures and explaining at the same time the severe symptoms of these infections (skin and internal organs bleeding, ulcerations and necrosis).

The present study has revealed among the VF of halophilic vibrios, the high frequency and resistance to a broad range of Na concentrations and pH as well, of the enzyme mucinase especially in *V. alginolyticus* (Fig. 5), *V.vulnificus* (Fig. 6), *V.parahaemolyticus* (Fig. 4) and *V.metschnikovii* (Fig. 2).

The high resistance to high acid pH of certain virulence factors (i.e. mucinase) could be considered a similar aspect to that described for gastric *Campylobacter* infections, able to destroy the protective mucin layer of the gastric wall. This aspect could also have practical

importance for public health, demonstrating the risk of raw shellfish outbreaks (as consequence of ingestion of seafood processed by vinegar).

Microorganisms are dependent on successful colonization of the host in order to survive and multiply. A major impediment to this process is the mucosal barrier, that exists in a secreted or membrane bound form. In order to breach this barrier, microorganisms have the ability to produce a range of hydrolyzing enzymes called mucinases, responsible for mucus degradation (a complex of high weight molecules that are the major non-aqueous components of the mucous gels).

The mucins are glycoproteins-targets for many different proteolytic and glicolytic enzymes. Partial or complete degradation of mucin molecules by microbial enzymes is signifying a fundamental step in disruption of defensive mucosal barriers as these constitute direct interfaces between internal and external media (36).

The possible contribution of mucin degrading enzymes to the pathogenesis of infections is therefore not to be underestimated.

Among mucin degrading enzymes there must be considered glycosidases, proteases, mucinases and sulphatases, playing the role of virulence factors and with possible effect on immune function (by degrading the host defense components of mucus such as IgA or lactoferrin with role of physical barrier) and promoting bacterial adhesion and colonization.

Mucinase and phospholipase (lecithinase) could promote tissue erosion and ulceration by destroying the mucus integrity and inducing lipid peroxidation.

Concerning the implication of iron as an essential compound for the growth and virulence of bacteria, this ion requires a siderophore for being acquired by microorganisms, from the extracellular environments in the purpose to be used for bacterial growth and virulence potential. In this purpose, aesculin (a plant glucoside) can be hydrolyzed by means of bacterial aesculinase to aesculetole (14) which is playing the role of siderophore.

Our results with reference to the inhibitory effect (decreasing up to disappearance) of glucose high concentration upon the expression of some VF come into agreement with Croxatto (15) and Saier et al. (33) considerations who pointed out that the catabolic repression is characterized by an inhibiting effect upon the expression of some (sets of non-related) genes codifying for catabolic enzymes in cases when glucose or other nonspecific hydrocarbonate substrata are present in the cultivation media.

However, the increasing expression of other VF in the presence of high glucose concentrations could explain the severe infections developed by the respective bacteria (i.e. *V. vulnificus* as resulting from our study) in hyperglicemic patients.

Conclusions

The present results indicate the ability of nonhalophilic and halophilic vibrios for adaptation in stress conditions (low temperature, large spectrum of NaCl, different glucose concentrations and pH), surviving and preserving their virulence potential, therefore signifying an environmental reservoir of virulence, promoting human and fish aquacultures infections.

The present study pointed out the significance of the Danubian aquatic environment as reservoir of virulence potential, demonstrating the necessity of further investigations of these aspects in the purpose to identify the risk factors of environment colonization by microorganisms with pathogenic and epidemiogenic potential and with possible implications in human pathology in certain geographical area of the country.

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