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Virulence factors and antimicrobial resistance in environmental strains of *Vibrio alginolyticus*

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Summary. *Vibrio alginolyticus* has acquired increasing importance because this microorganism may be pathogenic to aquatic animals and humans. It has been reported that some *V. alginolyticus* strains carry virulence genes derived from pathogenic *V. cholerae* and *V. parahaemolyticus* strains. In this work *V. alginolyticus* was isolated from oyster samples acquired from a food-market in Mexico City. Thirty isolates were identified as *V. alginolitycus*. Strains showed β-haemolysis and proteolytic activity and produced a capsule. Strains displayed swimming and swarming motility and 93.3% of them produced siderophores. Several genes encoding virulence factors were detected using PCR amplification. These included *proA*, *wza*, *vop*D, *vop*B, *hcp*, *vas*H and *vgr*G genes, which were present in all strains. Other genes had a variable representation: *tdh* (86.6%), *lafA* (96.6%), *pvs*A (62%) and *pvu*A (16%). The *trh* gene could not be amplified from any of the strains. The antimicrobial resistance profile revealed that more than 90% of the strains were resistant to beta-lactams antibiotics, 60% to cephalotin, 45% to amikacin, 16% to cephotaxime, and 10% to pefloxacin, while 100% were susceptible to ceftriaxone. The *V. alginolyticus* strains isolated from oysters showed multiple resistance to antibiotics and several virulence factors described in well-characterized pathogenic vibrios. [**Int Microbiol** 19(4):191-198 (2016)]

Keywords: *Vibrio alginolyticus* · secretion system · virulence factors · capsular polyssacharides · oysters

Introduction

According to the Center for Disease Control and Prevention (CDC), in recent years the *Vibrio* genus has caused several intestinal as well as extra intestinal disorders; twelve species have been described as pathogenic for humans, including *Vibrio cholerae*, *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio mimicus* and *Vibrio alginolyticus* and some of these species have been also recognized as causing *Vibrio*sis in marine vertebrates and invertebrates [36]. In 2014 several reports on *Vibrio* infections came from the coastal regions of the Gulf of Mexico with 34% of the cases [6].

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The pathogenicity mechanism of *V. alginolyticus* is not yet fully understood, however several virulence factors have been described. *Vibrio alginolyticus* possesses polar and lateral flagella [7,20]. Flagella have been associated with adhesion to surfaces, formation of biofilm and *swimming* and *swarming* [17]. *V. alginolyticus* produces hydroxamate type siderophores and three hemolysins; Thermostable Direct Hemolysin (TDH), Thermostable Related Hemolysin (TRH) and Thermolabile Direct Hemolysin (TLH) [31,41,43,47]. Studies have shown that the *vppC* gene is a key virulence gene contributing to the pathogenicity of *V. alginolyticus*. This gene has been used to distinguish between *Vibrio* strains by PCR amplification [11,23,24].

Some reports about the pathogenicity of *V. alginolyticus* indicate that enzymes with proteolytic activity are important virulence factors; one of them is a serine protease, which has a lethal effect on fish [16,21]. The capsule is considered an important virulence factor in some species of genus *Vibrio*, acting as a shield against the complement and blocks phagocytosis [16,34]. The type III secretion system (T3SS) has not been fully described in environmental strains of *V. alginolyticus*. This is a Sec-independent pathway in which secretion occurs in one step from the cytosol to the outside of the cell, playing a central role in the pathogenicity of many Gram-negative bacteria [29,39].

In *V. cholerae,* the type VI secretion system (T6SS) plays an important function, allowing this organism to compete with other bacteria and escape from phagocytic cells, enabling it to persist in infected humans and in the environment [8,27]. Proteins Hcp and VgrG have been recognized as key components of an active T6SS [25]. Studies on the T6SS in *V. cholerae* suggest that the effector proteins VgrG and Hcp are important not just as secretion products but also as a structural part of the secretion apparatus [38].

Vibrio sp. isolated from environmental samples have shown resistance to amoxicillin, ampicillin, cefuroxime, rifampin, and streptomycin, chloramphenicol, tetracycline, trimethoprim and nalidixic acid, neomycin and amikacin [22]. It has been reported that strains of *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus* and *V. harveyi* isolated from food showed ampicillin resistance almost 50% of the isolates [19,30]. Due to the scarce information existing for *V. alginolyticus*, this study aims to provide information about some virulence factors in strains isolated from bivalve mollusks.

Materials and methods

A total of 30 oysters samples were acquired at "La Nueva Viga" market in the Central de Abastos of Mexico City. Samples were transported frozen in individually labeled and sealed plastic bags to avoid contamination. The time between sample collection and analysis was approximately 24 h.

Isolation and phenotypical identification of *Vibrio alginolyticus*. *Vibrio alginolyticus* strains were isolated as described in the Bacteriological Analytical Manual of the Food and Drug Administration [10]. Strain identification was done using The API 20E system (BioMerieux). Strains *V. alginolyticus* ATCC 29397 and *V. parahaemolyticus* ATCC 17803 were used as positive controls in all phenotypic tests.

Determination of hemolytic activity and proteolytic activity. For the detection of hemolysis, strains were streaked on blood agar with 5% sheep erythrocytes. Plates were incubated for 24 h at 37 °C, and after this time, the appearance of hemolytic zones was registered [28]. For the proteolytic activity, *V. alginolyticus* strains were inoculated on BHI agar plates containing 3% NaCl and incubated at 37 °C during 24 h. Afterwards, a single colony was selected and streaked on 5% milk agar containing 3% NaCl and incubated at 37 °C during 24 h.

Swimming and swarming motility. Swimming and swarming tests for the *V. alginolyticus* strains were done according to Böttcheret al. [3].

Siderophores production. To induce siderophore production, *V. alginolyticus* strains were grown in nutrient broth at 37 °C during 24 h. Afterwards, strains were spread onto Cromo Azurol S Agar plates (CAS) [4] and incubated at 37 °C during 24 h.

Capsule production. A colony of *V. alginolyticus,* which had been cultured on Glycerol Agar to enhance capsule production [1], was mixed with a drop of Congo Red in the center of a slide to make a smear, the slide was allowed to air dry and then it was covered with capsule mordent for one minute. The smear was washed with distilled water and dried, the smear was observed under the microscope with the immersion objective [34]. To observe the presence of capsule using transmission electron microscopy (TEM), the Hébert et al. [18] methodology was followed with a minor change. In this experiment 1 M sodium cacodylate buffer at pH 7.2 were used instead of 0.2 M s-Collidine Buffer at pH 7.4. *Klebsiella pneumonie* ATCC 700603 was used as a positive control.

Genetic analysis. Strains were grown in Luria–Bertani broth containing 3% NaCl and incubated at 37 °C for 24 h. Chromosomal DNA from strains was extracted using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). The amplification mix was done with 12.5 µl of Master mix 2X (Thermo Scientific), 7.5 µl of water nuclease-free, 1 µl of reverse primer and forward primer (10 µm of each primer) and 100 ng of template DNA in a final volume of 25 µl. Amplifications were performed in a thermal cycler Techne (TC-3000G, Staffordshire, UK) with the primers shown in table 1. All amplifications included an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s (1 min for *vppC*, *vopB* and *vopD*), and extension for 1 min at 72 °C (30 s for *vppC*, *vopB* and *vopD*). Hybridization temperatures and the expected amplicon sizes are included in Table 2. Genomic DNA from *V. alginolyticus* ATCC 17749 was used as a positive control. *Vibrio parahaemolyticus* ATCC 17803 was used as a negative control for identification *vppC* gene and *E. coli* ATCC 25922 as a negative con-

trol for identification *tdh, trh, proA, pvsA, pvuA, wza, lafA, vop D, vopB, hcp, vasH* and *vgrG* genes.

Fragments corresponding to T3SS and T6SS encoding genes were sequenced. Sequence analysis was carried out with software DNASTAR version 7.1 (Lasergene, Madison, WI, USA), Seaview 4.32, and the search for regions of local similarity between our sequences and those deposited in the GenBank were done using the BLAST algorithm at the NCBI webpage [http://blast.ncbi.nlm.nih.gov/Blast.cgi].

Transcriptional expression of *vasH, vgrG* and *hcp* genes. To demonstrate the expression of selected T6SS genes from *V. alginolyticus,* strains were inoculated in BHI broth containing 3% NaCl and incubated at 25 and 37 °C for 12 h with shaking. Subsequently, total RNA was obtained with the commercial kit RNeasy Bacteria Mini Kit (Qiagen), following the manufacturer recommendations. The concentration of the isolated RNA was measured using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Finally, a reverse transcription-polymerase chain reaction assay (RT-PCR) was performed using the RT-PCR One step kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer recommendations. cDNA synthesis was carried out during 15 min at 50 °C, followed by an incubation at 94 °C for 2 min; PCR primers and amplification conditions used are stated in Tables 1 and 2. Gene *gyrB* was used as a constitutive gene. PCR products were separated by 2% Agarose gel electrophoresis and stained with ethidium bromide (Merck, Darmstadt, Germany). Amplicons were visualized under UV light in a gel-doc system (BioRad, Münich, Germany).

Antimicrobial resistance test. Antimicrobial susceptibility testing was made according to the Kirby-Bauer method [9]. *Vibrio alginolyticus* strains were grown in BHI broth with 3% NaCl, turbidity was adjusted at 0.5 of the McFarland nephelometer and were plated on Mueller Hinton agar with 3% NaCl, the pH was adjusted to 7.3 ± 0.1. The Multidiscs used were from Biorad cat. 7108280 and 7108180. The minimal inhibitory concentration was made by the macrodilution method using amikacin, cefotaxime, gentamicin, chloramphenicol, netilmicin, pefloxacin, ampicillin, dicloxacin, erythromycin and tetracycline. All strains was grown on BHI broth with 3% NaCl and *Escherichia coli* ATCC 25922 was used as control. Interpretation of the results was according to the document M45-A2 [9].

Statistical analysis. The correlation between antibiotic susceptibility and the presence of virulence genes was analyzed statistically using the Chi-Square test or Fisher's exact test and $P < 0.05$ was considered statistically significant. All tests were performed using GraphPad Prism 7 software.

Table 1. Primers used in this study

Target	Primer (5′→3′)	Reference
vppC	F:GTCGGATTCAGAGTCGGTATTTAG R: GTTCCACTGCCCACCAAACA	[11]
trh	F:AGGCAATTGTGGAGGACTATTG R: CTCTGATTTTGTGAAGACCGTAG	This study
tdh	F: CCCGGTTCTGATGAGATATTG R: ACCGCTGCCATTGTATAGTC	This study
proA	F: CCGGAATTCGAGCAATTCAC R: CACGAGGATCAGAACGAAGAT	[24]
lafA	F: GCTTTATCAATGCACACTAACT R: CAAGGTCGTTCATACGGTAA	This study
pvsA	F: AGCCGCCTTACTTTATCG R: CGAGACAATCGAAATTCG	This study
pvuA	F: AATTGCCTACATCCGAGG R: CAACATTCATGCGTAACTTG	This study
wza	F: AGCGGGTAACTGGGTGCATTCG R: CAGTAAGGCTCATGCCGCTACGATC	This study
vopD	F: GATAAAAATGGTGGAACGGG R:CGTTCTTCGGCTTGGTTT	This study
vopB	F: AARGGCGCAACGGACAG R: CAGGACGGCTTAACACCG	This study
vgrG	F: GAAGACGAAGCGAACCAAG R: ATTGAACCATCACTGTTCATCAC	This study
hcp	F: CAAGAAGCTCACGTTGATG R: TTGATTGAGTAGTAGTGCTCTTG	This study
vasH	F: AGTAGCATTGGCATATCRCC R: TCTTTGGCTGATCGCAGC	This study
gyrB	F:TCAGAGAAAGTTGAGCTAACGATT R: CATCGTCGCCTGAAGTCGCTGT	[27]

Results

*****Vibrio alginolyticus* isolation and identification.** From the 30 analyzed samples, 13 were positive for *V. alginolyticus*. Seventy isolates from these samples were presumptively classified as *V. alginolyticus* in biochemical tests. The appearance of 425 bp amplicon derived from the *vppC* gene confirmed 30 of these isolates. These strains were used for the next experiments. All *V. alginolyticus* strains showed β–hemolysis and proteolytic activity under the conditions tested; 93.3% of the strains (28/30) showed siderophore production and 100% of strains showed the presence of capsule with both the Congo Red staining. All studied strains displayed swimming motility, which is characterized by the spread of bacteria throughout the agar, resulting from a disordered migration throughout the agar surface. All strains were also positive for the swarming effect on agar plates.

Amplification of genes *tdh, trh, proA, pvsA, pvuA, wza, lafA, vop D, vopB, hcp, vasH* and *vgrG*. Table 3 shows the PCR amplification results of all the genes examined in this study. Among the 30 strains tested, 26 showed the presence of the *tdh* gene (86.6%), however, in none of them the *trh* gene could be amplified (data not shown). The *proA* and *wza* genes were amplified in all working strains

Table 2. Conditions used for the detection of genes

	vppC	[1]*vopD*, [2]*vopB*	[1]*lafA*, [2]*proA*	[1]*vgrG*, [2]*vasH*	*hcp*	[1]*pvsA*, [2]*pvuA*	*gyrB*	*trh*	*tdh*	*wza*
Hybridization	53 °C/ 1 min	52 °C/ 1 min	51°C/ 30 s	54 °C/ 30 s	55 °C/ 30 s	47 °C/ 30 s	58 °C/ 30 s	52.5 °C/ 30 s	47 °C/ 30 s	66 °C
Amplicon size (pb)	425	[1]400 [2]225	[1]283 [2]1	[1]532 [2]902	248	[1]467 [2]545	568	202	220	425

of *V. alginolyticus*. Gene *lafA* was amplified in 96.6% of the strains. Genes *pvsA* and *pvuA* were amplified in 62% and 19% on strains of *V. alginolyticus*, respectively.The expected length of the gene fragment amplified was obtained in 100% of the strains tested for the *vopD* and *vopB* genes belonging to the T3SS and the same was true for *vgrG*, *hcp* and *vasH* genes of the T6SS. Amplicons were sequenced and the resulting sequences were deposited in the GenBank database under accession numbers KT971351 for *Hcp*, KT934265 for *vgrG*, KT971348 for *vasH*, KT971350 for *vopB* and KT971349 for *vopD*.

Transcriptional expression of determinant genes for the T6SS function. For the expression analysis of genes *vasH, vgrG* and *hcp,* belonging to the T6SS, RNA from the *V. alginolyticus* strains grown at 25 or 37 °C was retrotranscribed and PCR amplified using primers shown in Table 1. The *gyrB* gene was used as an internal RT-PCR control. Figure 1 shows the RT-PCR products obtained by after growing the bacteria at 25 °C. Results indicate that expression of key genes of the T6SS takes place under this incubation temperature but it does not occur at 37 °C (data not shown).

Antimicrobial resistance. All the strains tested were sensitive to ceftriaxone, but resistant to carbenicillin and dicloxacillin. The percentage of strains resistant to ampicillin was 96%, 60% of the strains was resistant to cefalotin, 45% to amikacin, 16% to cefotaxim, 10% to trimethoprim-sulfamethoxazole, 3% to chloramphenicol, 38% gentamicin and 84% to tetracycline.

The strains that showed antimicrobial resistance by disc diffusion method were selected for the minimum inhibitory concentration assay. 100% of the strains (14/14 strains) showed a resistance to amikacin greater than 128 µg/ml, 80% (4/5 strains) were resistant to ≥ 32 µg/ml of cefotaxime, 47% (5/11 strains) were ≥ 16 µg/ml of Gentamicin, 100% (1/1) were ≥ 16 µg/ml of chloramphenicol, 63% (17/27 strains) were ≥ 32 µg/ml of Netilmicin, 12.5% (1/8 strains) were ≥ 4 µg/ml of Pefloxacin, 96% (26/27) were resistant to ≥ 16 µg/ml of Ampicillin, 100% (29/29 strains) were ≥ 64 µg/ml of Dicloxacin, 72% (8/11 strains) were resistant to gentamicin ≥ 8 µg/ml and 92% (23/25 strains) were resistant to ≥ 16 µg/ml of tetracycline. In the present study, it was shown that the *tdh* virulence gene has a significant association with amikacin ($P = 0.0445$); the *pvsA* with tetracycline ($P = 0.0455$); *pvuA* with amikacin ($P = 0.0104$) and with gentamicin ($P = 0.0372$); and *lafA* with tetracycline (Table 4).

Discussion

The vibriosis caused by *V. alginolyticus* on marine organisms is characterized by the appearance of hemorrhages, necrosis and ulcers on the injured skin, as well as sepsis. These clinical signs are caused by the virulence factors of the microorganisms, such as the production of multiple extracellular products (ECP), amongst them siderophores, hemolysins and proteases

Table 3. Detection and expression of genes in tested strains

	vvpC	*tdh*	*trh*	*proA*	*pvsA*	*pvuA*	*wza*	*lafA*	*vopD*	*vopB*	*hcp*	*vasH*	*vgrG*
Detection	30	26	0	30	18	6	30	29	30	30	30	30	30
Expression											30	30	30

[2]. In this study, all strains displayed β-hemolytic activity. Additionally, PCR was performed for the detection of the *tdh* and *trh* genes, coding for the TDH and TRH hemolysins respectively. TDH and TRH are the major recognized virulence factors in *V. parahaemolyticus* [5,40]. The presence of *tdh* gene is variable in *V. alginolyticus*. In the study reported by Weiet al. [46] it was not possible to amplify the *tdh* gene from any of the 72 *V. alginolyticus* strains tested, which contrasts with our results. On the other hand, Ren et al [35] detected in *V. alginolyticus* the *tdh* gene in 6 of nine pathogenic strains from a total of 31 strains (19.4%), the pathogenic strains caused the dead after the challenge in fish Orange-spotted grouper (*Epinephelus coioides*). It is important to indicated that foodborne infections caused by *V. parahaemolyticus* harboring the tdh^+ or tdh^+/trh^+ genes are relatively more common than those produced by *V. parahaemolyticus* tdh^-/trh^+ strains [37].

On the qualitative assay of proteolytic activity, the 30 strains identified as *V. alginolyticus* produced extracellular proteases and all of them contained the *proA* gene coding for alkaline serine protease. Previous works have reported that the proteases produced by *V. alginolyticus* have a toxic effect on *Ostrea edulis* larvae [35,43] and a lethal effect on *Penaeus japonicus* [15]. A study reported that a mutation in the *proA* gene, leads to changes in motility, colony morphology and extracellular polysaccharide production in *V. alginolyticus* [15]. In this study, we demonstrated that the *proA* gene is widely distributed amongst *V. alginolyticus* strains from environmental origin, which suggests, according to the literature, that these strains have the ability to affect the health of the fish, and maybe humans too.

Bacterial mobility, a shared mechanism among numerous microorganisms, is essential for pathogenic bacteria during their initial invasion and colonization process, in this case, swarming a coordinated rapid migration of bacterial cells

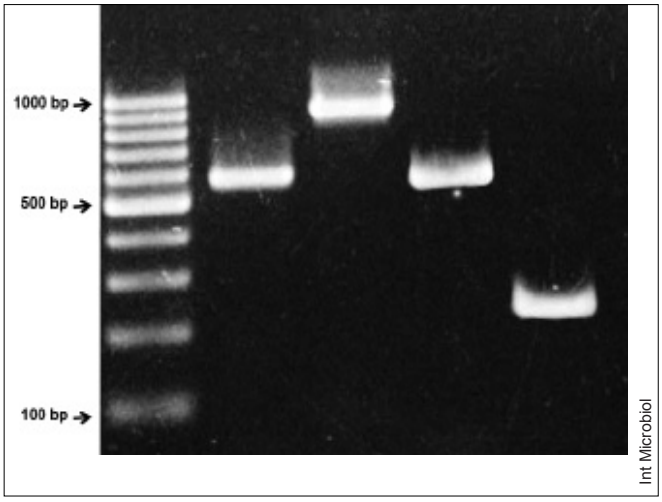

Fig. 1. RT-PCR detection of genes belonging to the SST6 of *V. alginolyticus* strains. Lane 1, 100 bp molecular weight marker; lane 2, *gyrB* (housekeeping gene); lane 3, *vasH*; lane 4, *vgrG* and lane 5, *hcp*.

across surfaces is an important but poorly understood aspect of bacterial [44]. In our work, all strains of *V. alginolyticus* showed swarming motility after inducing the expression of the lateral flagella by growth on solid medium. Lateral flagella allow microorganisms to travel on highly dense environments. Therefore, colonization by this microorganism when present on viscous tissues will be favored [7]. This kind of move has been associated with virulence for pathogens such as *Proteus mirabilis*, *Salmonella typhimurium* and *Clostridium septicum*. In other hand, swarming cells can also exhibit elevated antibiotic resistance compared with nonmotile populations [3].

Gene *lafA* was found in 96.6% of strains. This gene belongs to a lateral flagella gene system [32] and we can assume that *lafA* gene is expressed because all strains had swamming motility. *V. alginolyticus* was reported to be one of the most invasive and highly fatal fish pathogens in the South China

Table 4. Statistical analysis for determining possible relationship between antibiotic resistance and *tdh, pvsA, pvuA* and *lafA* genes in *Vibrio alginolyticus* strains

Antimicrobial agents	*tdh*	*pvsA*	pvuA	*lafA*
Ampicillin	$P = 0.6899$	$P = 0.4063$	$P = 0.6111$	$P = 0.8502$
Tetracycline	$P = 0.1143$	$P = 0.0455$	$P = 1.0000$	$P = 0.0229$
Amikacin	$P = 0.0445$	$P = 0.1138$	$P = 0.0104$	$P = 0.3091$
Gentamicin	$P = 0.1021$	$P = 0.5474$	$P = 0.0372$	$P = 0.4390$

P: probability value based on Chi-square test.

Sea, possessing two types of unique flagellar systems [44].

The siderophores produced by the *V. alginolyticus* strains are of the hydroxamate type [45], which was evident from the yellow halo surrounding the colony. In order to corroborate the presence of siderophores on *V. alginolyticus* strains, *pvsA* and *pvuA* genes were amplified in 62% and 19% on strains of *V. alginolyticus*, respectively. One study demonstrated that *pvsA* and *pvsD* genes are involved in the siderophore biosynthesis. They also showed that PvuA protein participates in the siderophore utilization process in *V. alginolyticus* MVP01 strain [41].

Studies on the genetics of polysaccharide biogenesis for the genus *Vibrio* are scarce. In *V. vulnificus* the capsular polysaccharide is a major virulence factor that has been the target an intensive study [13].The presence of capsule and the amplification *wza* gene were seen in 100% of the strains of *V. alginolyticus* studied in this work. A role of the capsule is to form a hydrated gel structure around the bacterium, protecting the organism from desiccation effects [13], but the main role of capsule in pathogenesis is to help bacteria resist the action of the complement, acting as a barrier that masks structures that can potentially activate the alternative pathway [4,13]. To our knowledge this is the first report showing the presence of capsule in environmental strains of *V. alginolyticus.*

Secretion systems. Genes *vopB* and *vopD* were amplified in all *V. alginolyticus* strains. These genes encode proteins involved in translocating effector proteins via the type III secretion system (T3SS), they are considered essential in the structure and performance of system. Several studies have demonstrated that T3SS is part of the mechanism of pathogenicity in many bacteria [12,29,39] and other studies have suggested that *V. alginolyticus* might carry out extensive gene exchange of these genes with environmental bacteria and thus serve as a reservoir of virulence genes from *V. cholerae* and *V. parahaemolyticus* [32,38].

The SSTIII has not been fully studied in *V. alginolyticus* but in other bacteria such as *V. parahaemolyticus* has been shown to be involved in toxicity towards cell cultures as well as in lethal activity in a mouse supplied intraperitoneally [42]. It has also been shown that this secretory system plays an important role in allowing it to escape from phagocytosis as well as being an interbacterial virulence factor that gives the ability to survive in certain ecosystems where there is greater competition interspecies [21].

Galan et al. [14] mention that the effector proteins of the SSTIII in *V. parahaemolyticus* are involved in the nuclear condensation and fragmentation of the DNA of the white cell, provoking cell death. So they are important not only as a secretion product, but also as a structural part of the SSTIII. On the other hand, the presence of orthologs of the 18 SSTIII genes in the three main vibrios (*V. cholera*, *V. parahaemolyticus* and *V. mimicus*) has been demonstrated. The common objective of SSTIII effectors include the actin cytoskeleton, innate immune signaling and autophagy, these systems can be regulated according to the specific needs of the pathogens [17].

Fragments of the *hcp, vgrG* y *vasH* genes were amplified in all the environmental strains of *V. alginolyticus*. These genes code for effector proteins essential for the structure and functioning of the secretion system type VI. Results indicate that the essential genes of the secretion system type VI are distributed in all environmental strains of *V. alginolyticus* and that it is likely that this virulence factor may be part of their pathogenicity mechanism. Results from the expression at the transcriptional level of the essential genes of the T6SS showed that the highest expression levels were clearly achieved at 25 °C, which suggests that the type VI secretion system is expressed in bacteria in their ecological niche. This is consistent with the report by Salomon et al. [40] who evaluated the level of expression of the T6SS in *V. parahaemolyticus* at 25 °C, which is a similar condition to the one found at its ecological niche. To our knowledge, this is the first study showing that the temperature may contribute to control the expression of essential genes of the T6SS in environmental *V. alginolyticus* strains. However, we do not know what other environmental signals could control the expression of the T6SS system, especially during infection of the host, such as pH, concentration of bile salts and oxygen. It has been shown that at least six effector proteins can be translocated by the T6SS and that all have a bactericidal activity, allowing *V. alginolyticus* to persist in marine environments [40]. However, it will be important to demonstrate the activity of these effectors during the infection process.

Antimicrobial resistance. All strains showed different antibiotic resistance patterns, but we weren't able to correlate this data with the use of antibiotics in aquaculture in Mexico, mainly due to the fact that there is no official registry of its use. However, the results obtained in this study correlate to those obtained by other authors, where 95% of *V. alginolyticus* showed resistance to carbenicillin, ampicillin, penicillin,

and cefalotin [30]. *V. alginolyticus* is generally resistant to penicillin and vancomycin but sensitive to ciprofloxacin, chloramphenicol, aminoglycosides and beta-lactams [26]. It has been pointed out in other studies that *V. alginolyticus* is sensitive to trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, gentamicin, quinolones and first generation cephalosporin [26]. High levels of resistance were found on the minimum inhibitory concentration assay to some antibiotics like amikacin (≤ 128 μg/ml), cefotaxime (≤ 64 μg/ml) and netilmicin (≤32 μg/ml). While low levels of resistance were found on chloramphenicol (≤16 μg/ml) and gentamicin (≤μg/ml). Similar results have been published by Ottaviani et al. [33] and Lajnef et al. [23] although there are no previous studies reporting high levels of resistance to amikacin and cefotaxime**.** There exists a correlation between the increase in antibiotic resistant bacteria and the indiscriminate use of them in fish farms which puts at risk, not only aquaculture but also human health since many of these products are consumed raw.

In this work we documented the presence of several virulence factors in environmental *V. alginolyticus*. As shown for other pathogenic vibrios, these determinants may enable the microorganism to invade the host, cause tissue damage in order to access nutrient sources required for its growth and propagation. However, it is necessary to perform extensive studies on the virulence factors and the spread of antibiotic resistance genes on *V. alginolyticus* in order to find out more about its potential risk to public health.

Competing interests. None declared.

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