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Review

Role of bacterial secretion systems and effector proteins – insights into *Aeromonas* pathogenicity mechanisms*

Joanna Matys[1]✉, Anna Turska-Szewczuk[2] and Anna Sroka-Bartnicka[1,2]

[1]Department of Biopharmacy, Medical University of Lublin, Lublin, Poland; [2]Department of Genetics and Microbiology, Institute of Microbiology and Biotechnology, Maria Curie-Sklodowska University, Lublin, Poland

Gram-negative bacteria have developed several nanomachine channels known as type II, III, IV and VI secretion systems that enable export of effector proteins/toxins from their cytosol across the outer membrane to target host cells. Protein secretion systems are critical to bacterial virulence and interactions with other organisms. *Aeromonas* utilize various secretion machines, e.g. two-step T2SS, a Sec-dependent system, as well as one-step, Sec-independent T3SS and T6SS systems to transport effector proteins/toxins and virulence factors. Type III secretion system (T3SS) is considered to be the dominant virulence system in *Aeromonas*. Activity of bacterial T3SS effector proteins most often leads to disorders in signalling pathways and reorganization of the cell cytoskeleton. There are also scientific reports on a pathogenicity mechanism associated with the host cell apopotosis/pyroptosis resulting from secretion of a cytotoxic enterotoxin, i.e. the Act protein, by the T2SS secretion system and an effector protein Hcp by the T6SS system. Type IV secretion system (T4SS) is the system which translocates protein substrates, protein-DNA complexes and DNA into eukaryotic or bacterial target cells. In this paper, contribution of virulence determinants involved in the pathogenicity potential of *Aeromonas* is discussed. Considering that the variable expression of virulence factors has a decisive impact on the differences observed in the virulence of particular species of microorganisms, it is important to assess a correlation between bacterial pathogenicity and their virulence-associated genes.

Key words: *Aeromonas,* virulence factors, bacterial secretion systems, effector proteins

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✉e-mail: joanna.matys@umlub.pl

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Abbreviations: ADPRT, ADP-ribosyltransferase; GCAT, cholesterol acyltransferase; GAP, GTPase-activating protein; OMVs, outer membrane vesicles; OMP, outer membrane proteins; LPS, lipopolysaccharide; PFTs, pore-forming toxins; T2SS, type II secretion system; T3SS, type III secretion system; T4SS, type IV secretion system; T6SS, secretion system

INTRODUCTION

Bacterial pathogens have evolved a multitude of strategies against prokaryotic competitors and eukaryotic hosts to colonize, invade, and overcome the host immune response (Sha *et al.*, 2005; Fernandez-Bravo & Figueras, 2020). One of important prokaryotic cell functions is protein secretion, which comprises transport of proteins from the cytoplasm to the extracellular medium and/or directly into other bacteria or eukaryotic cells. Protein secretion has an essential impact on these strategies, used by many bacterial pathogens (Maffei *et al.*, 2017; Burdette *et al.*, 2018; Sana *et al.*, 2019). Since bacteria form a variety of biotic associations, such as biofilms or pathogenic associations with larger host organisms (Donlan, 2002; Bogino *et al.*, 2013), the role of protein secretion in modulating all of these interactions has been an important focus in the area of bacterial pathogenesis (Tseng *et al.*, 2009; Nazir *et al.*, 2017).

Many proteins secreted by pathogens, such as toxins and effector proteins, contribute to increased adhesion of microorganisms to eukaryotic cells and to direct disruption of target cell functions playing a role in promoting their virulence (Brodsky *et al.*, 2010). These proteins can be transferred from the bacterial cytoplasm into host cells or host environment *via* a variety of mechanisms, usually involving dedicated protein secretion systems, which are molecular machines translocating effector proteins across the host plasma membrane (Holland, 2004; Abby & Rocha, 2017; McQuade & Stock, 2018; Meuskens *et al.*, 2019). Bacterial pathogens use secretion devices in a number of processes that are essential for their growth. These secretory nanomachines fulfil a prominent role in pathogenic or symbiotic interactions between "invaders" and their hosts or in formation of microbial communities (Galan & Waksman, 2018). Gram-negative bacteria have developed a wide variety of protein secretion apparatuses (known as type II, III, IV, and VI secretion systems) that facilitate export of infection-related proteins through the inner and outer membrane (Depluverez *et al.*, 2016; Kubori, 2016; Abby & Rocha, 2017; Jana & Salomon, 2019).

Pathogenic bacteria are capable of causing diseases in susceptible hosts through the activity of multiple virulence determinants that work individually or in combination. Effector proteins secreted *via* these systems are usually critical for bacterial virulence, e.g. loss of T3SS is sufficient to render the bacteria completely avirulent. Evaluation of distribution of the virulence-associated genes that encode various effector proteins and toxins and their correlation with bacterial virulence could provide valuable insights into bacterial pathogenicity (Wu *et al.*, 2008; El-Bahar *et al.*, 2019; Reyes-Rodriguez *et al.*,

2019; Talagrand-Reboul *et al.*, 2020). This review presents a concise summary of the secreted effectors/toxins' contribution to the virulence potential of *Aeromonas*. Since expression of virulence determinants has a decisive impact on the differences observed in the virulence of particular species of microorganisms, it is necessary to study the correlation between virulence genes and bacterial pathogenicity. The role of bacterial secretion pathways (T2SS, T3SS, and T6SS) used to efficiently infect the host is discussed as well.

SECRETED VIRULENCE FACTORS IN *AEROMONAS*

Aeromonas, which are representatives of Gram-negative bacteria, are found in aquatic environments worldwide (Evangelista-Barreto *et al.*, 2010; Soto-Davila *et al.*, 2019). These rods are mostly pathogenic to poikilothermic animals, including amphibians, fish, and reptiles. These opportunistic pathogens provoke a large variety of fish diseases, which particularly affect cultured salmonids and cyprinid species, causing ulcers, haemorrhages, septicemias and furunculosis (the latter concerns diseases caused by *Aeromonas salmonicida* subsp. *salmonicida* in salmonids) (Dwivedi *et al.*, 2008; Dworaczek *et al.*, 2019). In humans, they can cause wound infections, bacteraemia, gastroenteritis, and less frequently hepatobiliary infections, respiratory infections, urinary tract infections, and peritonitis. These diseases are usually more severe in immunocompromised than immunocompetent individuals (Wahli *et al.*, 2005; Tang *et al.*, 2014; Praveen *et al.*, 2016; Abd-El-Malek, 2017; Duman *et al.*, 2018).

The pathogenicity of *Aeromonas* was found to be multifactorial (Turska-Szewczuk *et al.*, 2014; Rasmussen-Ivey *et al.*, 2016; Reyes-Rodriguez *et al.*, 2019) and attributed to a wide range of virulence-related factors (Albert *et al.*, 2000; Igbinosa *et al.*, 2012; Tomas, 2012; Turska-Szewczuk *et al.*, 2013), including structural components, i.e. polar (fla) and lateral flagella (laf), pili, capsules, the S-layer, outer membrane proteins (OMP), and lipopolysaccharide (LPS). Moreover, interaction between such pathogenic bacteria as *Aeromonas* and host cells is produced by their extracellular components and toxins, including haemolytic toxins, such as: 1) aerolysin with haemolytic and cytolytic properties (Aer), 2) cytotoxic enterotoxin (Act) with multiple biological activities, including ability to lyse red blood cells and destruct tissue culture cell lines (Chopra *et al.*, 2000), 3) thermolabile (Alt), and thermostable (Ast) cytotonic enterotoxins (Alt causes elevation of cyclic AMP and prostaglandin levels in intestinal epithelial cells, Ast possess similar properties to Alt) (Albert *et al.*, 2000), 4) serine protease (Ser) with extracellular proteolytic activity, 5) elastase (Ela) with caseinolytic and elastolytic activities (Rasmussen-Ivey *et al.*, 2016), 6) glycerophospholipids, such as cholesterol acyltransferase (GCAT), which attacks membrane phospholipids and leads to lysis of fish tissues (Tomas, 2012), and secretion systems (Ghenghesh *et al.*, 2014; Rasmussen-Ivey *et al.*, 2016; Dlamini *et al.*, 2018; Dworaczek *et al.*, 2019; Fernandez-Bravo & Figueras, 2020). To understand the pathogenicity of *Aeromonas*, the contribution

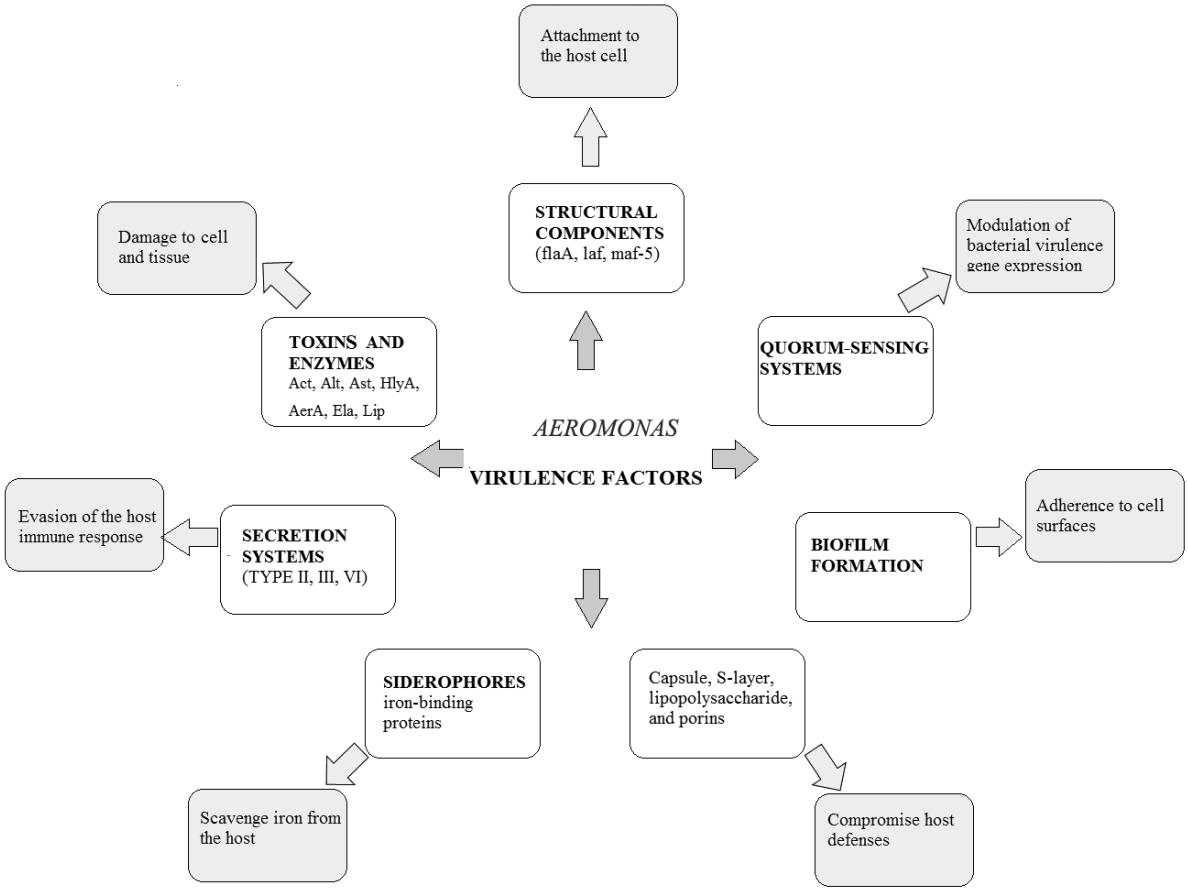

Figure 1. Role of the virulence factors contributing to the virulence of *Aeromonas*

of these factors should be defined. The role of determinants involved in various stages of infection mechanisms is presented in Fig. 1. Many researchers have focused their attention on the biochemical activity of extracellular enzymes that may lead to damage to the host cell, facilitating the pathogen to invade the host and cause infection (Sun *et al.*, 2016). Pathogenicity of *Aeromonas* results from a combination of various virulence determinants; therefore, continuous monitoring of the occurrence of several virulence-related determinants in isolates is crucial to clarify the pathogenesis and epidemiology of (Chacon *et al.*, 2003; Aguilera-Arreola *et al.*, 2005). Expression of genes (*alt, act, exoA, etc.*) that encode different toxins has been widely analysed in determining the pathogenicity potential of *Aeromonas* (Li *et al.*, 2011; Yi *et al.*, 2013). Wang et al. characterized the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* isolates. They suggest that *ahhl* was the most prevalent hemolysin gene in all of the examined *Aeromonas* isolates (Wang *et al.*, 2003). Another paper reports on a new functional hemolysin A gene (*hlyA*), which was found in a clinical isolate of *A. hydrophila* (Erova *et al.*, 2007). As proved by molecular characterization, the *hlyA* gene showed no homology with other known hemolysin and aerolysin genes identified in *Aeromonas* isolates. A role of this new hemolysin gene in the virulence potential of *Aeromonas* has been suggested.

Zhou and others (Zhou *et al.*, 2019) investigated virulence-related genes in *Aeromonas* strains obtained from patients suffering from extra-intestinal and intestinal diseases. The study of the distribution of virulence genes in the most common *Aeromonas* species *A. veronii, A. caviae, A. dhakensis*, and *A. hydrophila*, which have been isolated from clinical specimens, revealed 40 combinations of 10 genes, among which *alt/ela/lip/fla* was the most dominant combination in the isolates. The *alt/ela/li* and *act/ascF-G/fla* combinations dominated as well. Although different numbers and types of virulence genes associated with *Aeromonas* pathogenicity were shown, there was no significant correlation between these genes and the invasion potential both in intestinal and extra-intestinal infections (Zhou *et al.*, 2019). Similarly, Wu et al. showed no correlation between the presence of the virulence genes *aerA, hlyA, alt, ast,* and *ascFG* in *Aeromonas* isolates and the infection progress (Wu *et al.*, 2007). In contrast, Zhou and others (Zhou *et al.*, 2019) found that *A. hydrophila* species was more dominant in the case of extra-intestinal infections in comparison to intestinal infections. Moreover, they revealed predominance of this species, especially in patients with malignant tumors. As suggested by the authors, *Aeromonas* sp. should be considered as an infectious agent in immunosuppressed patients – in particular, those with gastroenteritis, liver cirrhosis, post liver transplantation, and malignancy. Other authors have studied the genetic diversity of *Aeromonas* species isolated from lake water (Khor *et al.*, 2015). Their results indicated involvement of multiple virulence genes (mainly: *ser, aer, fla, ela act,* and *aexT*), and among others *alt* and *ast* or their combination in *A. hydrophila* pathogenicity.

The latest research on correlation between the virulence genes and pathogenicity of *A. hydrophila* isolates has shown the presence of the *aer* gene in the majority of screened isolates (El-Bahar *et al.*, 2019). The *act* and *hlyA* genes were also identified, but in a much smaller number of the isolates. However, the *ast* gene was not found in any of the studied isolates. A direct relationship between the percentage of mortality and the genotype of the isolates was proposed based on the pathogenicity test. The mortality rates were ~60% for isolates in which the virulence genes aer^+ and act^+ were identified and ~ 70% for isolates with other genes: aer^+ and $hlyA^+$. A slightly smaller percentage (approx. 50%) was determined in isolates characterized by the presence of only one of the following genes: *act*, *aer*, and *hylA*. For isolates devoid of virulence genes, a mortality rate of approx. 20% was determined (El-Bahar *et al.*, 2019). Studies of the relationship between the presence of virulence genes and pathogenicity of *A. hydrophila* conducted previously by Li and others (Li *et al.*, 2011) showed a more frequent occurrence of the $aerA^+alt^+ahp^+$ virulence genotype in bacterial isolates from the diseased than from the healthy fish (Li *et al.*, 2011). A recent report of the comparative and evolutionary genomics of *Aeromonas* isolates has brought a breakthrough in understanding bacterial virulence (Talagrand-Reboul *et al.*, 2020). The authors have perfomed phylogenomic analyses of several virulence-associated genes: *aer/act, ast, alt, exoA, aexT, aexU,* and *lafA*. They suggested that the complexity of genes in terms of the varied gene organization, alternating evolutionary modes, and their unequal distribution could help to elucidate the difficulties in assessment of *Aeromonas* pathogenicity. Their observations were consistent with the existing assumption that Aer/Act is considered as the main enterotoxin involved in bacterial virulence. The novel accomplishment addressed the relationship between *aer/act* and *ser* genes that probably results from their functions. The *aer+/ser* pattern suggested that proteases (other than serine) may contribute to aerolysin activation or that Aer/Act is secreted but not matured in transmembrane protein complexes. The authors have demonstrated that the analysis of virulence-related genes should be conducted at the population level and studies performed on type strains cannot be generalized to the whole species (Talagrand-Reboul *et al.*, 2020).

Aeromonas spp. have evolved various secretion pathways to translocate virulence-related proteins to the extracellular medium or directly into the host cells. Type II, III, and VI (T2SS, T3SS, and T6SS, respectively) are well-known secretion systems identified in *Aeromonas* (Zhong *et al.*, 2019). The type II secretion system is related to the extracellular release of amylases, proteases, and aerolysin, as well as translocation of virulence determinants across the cell outer membrane (Sandkvist, 2001; Filloux, 2004; Li *et al.*, 2011; Korotkov *et al.*, 2012; Chernyatina & Low, 2019; Korotkov & Sandkvist, 2019). It is a double-membrane-spanning protein secretion system consisting of 12–15 various general secretory pathway (Gsp) proteins in multiple copies (Korotkov *et al.*, 2012). T3SS, which is considered as the dominant virulence system in *Aeromonas* (Origgi *et al.*, 2017; Fernandez-Bravo & Figueras, 2020), facilitates translocation of protein effectors across the plasma membrane into the host cell or secretion of pore-forming translocators that facilitate the transport of effector proteins (Chacon *et al.*, 2004; Sha *et al.*, 2007; Izore *et al.*, 2011; Rangel *et al.*, 2019). The type VI secretion system acts by inserting toxins into the host *via* valine-glycine repeat proteins and hemolysin-coregulated proteins (Wang *et al.*, 2011; Yang *et al.*, 2018; Fernandez-Bravo *et al.*, 2019). After secretion, these proteins exhibit antimicrobial pore-forming properties or remain as structural proteins (Bhowmick & Bhattacharjee, 2018). In recent years, research is particularly focused on the role of expression of genes encoding various toxins and secretion pathways in promoting *Aeromonas* virulence mechanisms (Dacanay *et al.*, 2006; Vanden Bergh & Frey, 2014; Soto-Davila *et al.*, 2019).

T2SS SECRETION SYSTEM AND EFFECTOR PROTEINS

Aeromonas infections are a result of complex molecular interactions between the pathogenic bacteria and the host cell, as indicated by proteins and toxins secreted into the extracellular environment. In *Aeromonas*, two types of hemolysins (α and β) differing in functional and physiological properties were determined (Epple *et al.*, 2004). Both have the ability to form pores in the membrane of the host cell, thus generating osmotic lysis (Cabezas *et al.*, 2017). Aerolysin, i.e. the prototype hemolysin of the genus encoded by the *aerA* gene (Fernandez-Bravo & Figueras, 2020), is secreted by the T2SS system (Korotkov *et al.*, 2012). Pore-forming toxins (PFTs) secreted by bacterial pathogens, such as *Aeromonas*, are major virulence factors used to modulate host cell apoptosis and cause auspicious infections (Bischofberger *et al.*, 2012; Escajadillo & Nizet, 2018). They are able to induce different types of host cell death, as demonstrated in numerous papers (Gonzalez *et al.*, 2011; Wiles & Mulvey, 2013; Ramirez-Carreto *et al.*, 2019). PFTs act by causing damage to the host cell membrane, which activates various signalling pathways in the cells (Podobnik *et al.*, 2017). Consequently, the permanent toxin-mediated membrane injury often leads to cell death (Gonzalez *et al.*, 2011). The crucial parameter determining the type of death (apoptosis, necrosis, or pyroptosis) is the concentration of toxins and cell types. Aerolysin (as PFT) is secreted as an inactive precursor (pro-aerolysin), which is transformed into aerolysin only after binding to high-affinity receptors on the target cell (Jia *et al.*, 2016). With its ability to form heptameric pores, this bacterial α-toxin leads to induction of membrane damage and cell death (Iacovache *et al.*, 2006; Wuethrich *et al.*, 2014; Escajadillo & Nizet, 2018). Cell apoptosis is associated with activation of caspases. Imre et al. have demonstrated that caspase-2 is necessary for PFT-mediated apoptosis and acts as an initiator caspase in *Aeromonas* aerolysin-mediated apoptosis (Imre *et al.*, 2012).

Aerolysin is a dimer, both in the crystal from and in solutions. Its main secondary structure is a β-sheet (more than 70% of the molecule) (Iacovache *et al.*, 2016). Conversion of proaerolysin to active aerolysin requires removal of approximately 43 amino acids from the C-terminus (Iacovache *et al.*, 2011). Cleavage can be achieved by proteases secreted by the bacteria or found in the digestive tract, e.g. trypsin, chymotrypsin, and furin (Abrami *et al.*, 1998). Three sites of cleavage have been identified: Lys-427 by trypsin, Arg-429 by chymotrypsin, and Arg-432 by furin. Aerolysin is a channel-forming toxin (Iacovache *et al.*, 2006) that binds to a specific receptor on the surface of target cells and oligomerizes to form heptamers, which can insert into the plasma membrane (Makobe *et al.*, 2012; Cirauqui *et al.*, 2017). Recently, a tripartite α-pore forming toxin (from the alpha helical CytolysinA family) has been identified in *Aeromonas hydrophila* (AhlABC). Structural analysis proved that the AhlABC toxin requires all three components for cell lysis. Wilson and others (Wilson *et al.*, 2019) proposed a bi-fold hinge mechanism of transition from the soluble to the pore form in AhlB structures, and a tetrameric assembly used by soluble AhlC to hide their hydrophobic residues related to the membrane. The type II secretion system, i.e. a well-known virulence mechanism in *Aeromonas*, is a trans-envelope apparatus used to deliver folded protein toxins to the surface and/or the extracellular environment of the cell (Howard *et al.*, 2019). Bacterial T2SS constitutes a large structure, including more than a dozen various proteins in multiple copies (Howard *et al.*, 2019). As demonstrated by Li and Howard (Li & Howard, 2010) in the secretion pathway of *Aeromonas hydrophila*, for assembly of type 2 secretion apparatus (secretin ExeD in the outer membrane), proteins ExeA and ExeB form an inner membrane complex which interacts with the peptidoglycan. While the peptidoglycan-ExeAB complex (PG-AB) is required for assembly of ExeD, the assembling mechanism remains unexplained. In their analysis of protein-protein interactions, Vanderlinde et al. suggested a putative mechanism by which the PG-AB complex facilitates the assembly of ExeD *via* a direct interaction between ExeB and ExeD (Vanderlinde *et al.*, 2014). Other researchers demonstrated a secretion defect in ExeAB mutants as a result of an inability to assemble ExeD secretin in the outer membrane. The location and multimerization of overproduced ExeD in these mutants indicated a role of the ExeAB complex in the transport of ExeD to the outer membrane (Ast *et al.*, 2002).

T3SS SECRETION SYSTEM AND EFFECTOR PROTEINS

Many authors have shown that the toxicity of T3SS causes mutations in both, the structural genes and effector proteins, thereby demonstrating that structural genes are necessary for the toxicity and virulence of *Aeromonas* species, such as *A. salmonicida* and *A. veronii* (Reyes-Rodriguez *et al.*, 2019). Effector proteins secreted *via* the type III secretion system, such as the serine/threonine kinase (AopO), tyrosine phosphatase (AopH), and ADP-ribosylating toxin (AexT) have been extensively studied. The activity of T3SS toxins leads to disorders in signalling pathways and reorganization of the cell cytoskeleton, thus contributing to phagocytosis impairment, as proven in numerous scientific reports (see Table 1) (Vanden Bergh & Frey, 2014; Origgi *et al.*, 2017; Soto-Davila *et al.*, 2019). The bi-functional ADP ribosylating – GTPase activating protein (AexT) is one of the effector proteins secreted *via* the T3SS system exerting a detrimental impact on the cell cytoskeleton and causing disruption of actin filaments in target cells (Fehr *et al.*, 2007; Vilches *et al.*, 2008). Increased mortality resulting from the presence of the AexU effector in a mouse model of *Aeromonas* infection has been reported based on comparative genomic and functional tests of virulence genes (Grim *et al.*, 2013). Characterization of virulence determinants in *A. hydrophila* proved that the virulence was associated with a combination of virulence factors: Act (T2SS effector), ExoA (exotoxin A), AexU (T3SS effector), and hemolysin co-regulated protein (Hcp, T6SS effector) or the presence of one of them (Grim *et al.*, 2014).

Effectors, secreted and translocated *via* the T3SS system, may induce host's inflammatory response (Asrat *et al.*, 2015; Soto-Davila *et al.*, 2019). This was demonstrated for the AoP effector, which inhibits the NF-κB signalling pathway contributing to modulation of the host's inflammatory response (Fehr *et al.*, 2006). Influence on the host's immune response, especially by down-regulation of the process, has been also suggested in the case of other protein effectors, such as Ati2, AopN, and ExsE (Vanden Bergh *et al.*, 2013). Origgi and others (Origgi *et al.*, 2017) demonstrated that infection of the rainbow trout (*Oncorhynchus mykiss*) with *Aeromonas salmonicida* strains with both, fully functional and secretion-impaired T3SS, was related to a strong immune suppression. The infection was also shown to be fatal only in the presence of fully functional T3SS, while the lack of T3SS was neither related to an immune suppression nor to death of the rainbow trout. These results confirmed

Table 1. *Aeromonas* **effector proteins with defined mechanisms of action**

Virulence-related genes	Secretion system	Mechanism of pathogenesis	References
act 2	T2SS	Cytotoxic enterotoxin with hemolytic, cytotoxic, and enterotoxic activities.	(Sha *et al.*, 2005)
		Induced upregulation of genes involved in immune responses (e.g., IL-8) and apoptosis (e.g., Bcl-2-like genes).	(Galindo *et al.*, 2003
		Activation of MEK1, JNK, ERK1/2, and c-Jun of the MAPK pathway; induced classical membrane blebbing; increased production of mitochondrial cytochrome C, caspase-3, -8, and -9 activation.	(Galindo *et al.*, 2004)
aexU	T3SS	Highly cytotoxic ADP-ribosyltranferase activity (ADPRT) to the host proteins.	(Braun *et al.*, 2002)
		Disruption of actin filaments and cell rounding, chromatin condensation, activation of caspase 3 and 9 (initiating host cell apoptosis).	(Sierra *et al.*, 2007)
		GTPase-activating protein (GAP) activity mainly responsible for the host cell apoptosis and disruption of actin filaments, and inhibition of NF-κB signalling.	(Sierra *et al.*, 2010)
		Ability to co-localize with β4-integrin resulting in cytotoxicity to the host cells.	(Abolghait *et al.*, 2011)
aexT	T3SS	Highly cytotoxic ADP-ribosyltranferase activity (ADPRT) to the host proteins.	(Braun *et al.*, 2002; Burr *et al.*, 2003; Vilches *et al.*, 2008)
		Promotes actin depolymerization.	(Braun *et al.*, 2002; Burr *et al.*, 2003; Vilches *et al.*, 2008; Vanden Bergh *et al.*, 2013)
		Bifunctional toxin, possesses a GTPase-activating domain and an ADP-ribosylating domain, which ADP-ribosylates both muscular and non-muscular actin. Both domains fulfill an independent role in the actin depolymerization and cell rounding.	(Burr *et al.*, 2005; Fehr *et al.*, 2006)
aopP	T3SS	Inhibitory activity against the NF-κB pathway (NF-κB pathway inhibition is highly proapoptotic after simultaneous cellular stimulation of the tumor necrosis factor-α).	(Dacanay *et al.*, 2006; Fehr *et al.*, 2006; Jones *et al.*, 2012)
		Induces apoptosis of a mammalian cell.	(Jones *et al.*, 2012)
aopH	T3SS	Putative tyrosine phosphatase.	(Beaz-Hidalgo & Figueras, 2013; Vanden Bergh *et al.*, 2013)
		Dephosphorylation of protein tyrosine residues in complexes of focal adhesion at the cellular membrane, leading to loss of the focal adhesion complex, changes in actin cytoskeleton, and prevention of phagocytosis.	(Najimi *et al.*, 2009; Broberg & Orth, 2010)
		Induces cytotoxicity in HeLa cells after transfection.	(Dacanay *et al.*, 2006)
aopO	T3SS	Remains poorly understood.	(Sha *et al.*, 2007)
		Putative serine/threonine kinase.	(Dacanay *et al.*, 2006; Vanden Bergh *et al.*, 2013; Menanteau-Ledouble & El-Matbouli, 2016; Bartkova *et al.*, 2017)
		Disturbs the normal distribution of actin in the host cell.	(Groves *et al.*, 2010)
ati2	T3SS	Suspected of having inositol polyphosphate phosphatase activity.	(Reith *et al.*, 2008)
		Ati2 is toxic to the host cell in a catalysis-dependent manner.	(Dallaire-Dufresne *et al.*, 2013)

Virulence-related genes	Secretion system	Mechanism of pathogenesis	References
vgrG1	T6SS	ADP-ribosylating toxin that is able to interrupt the host cell cytoskeleton and induce apoptosis in HeLa cells.	(Suarez *et al.*, 2008; Suarez *et al.*, 2010; Sha *et al.*, 2013)
		VgrG-2 negatively affects bacterial motility, while VgrG-3 works to positively influence motility; VgrG-1 does not directly influence motility, but is necessary for the activity of VgrG-2 and VgrG-3.	(Sha *et al.*, 2013)
		VgrG-1 negatively affects protease production in the absence of VgrG-2 and VgrG-3. All VgrGs play a role in biofilm formation (VgrG-2 and VgrG-3 are critical in regulating biofilm formation).	(Sha *et al.*, 2013) (Sha *et al.*, 2013)
		All VgrG proteins form a trimeric needle-like structure, allowing bacteria to penetrate the membrane and to transport effector proteins upon target cell contact.	(Bonemann *et al.*, 2010)
hcp	T6SS	Translocation to the target host cell is followed by apoptosis after caspase activation.	(Suarez *et al.*, 2008)
		Prevents phagocytosis.	(Suarez *et al.*, 2010)
		Inhibited proinflammatory cytokine production; induced immunosuppressive cytokines, such as interleukin-10 and transforming growth factor-b.	(Suarez *et al.*, 2010)
		Forms stacked hexameric rings to create a tube topped with VgrG tips (the needle-like complex allows bacteria to puncture the host cell and deliver effectors into the cell).	(Ho *et al.*, 2014; Russell *et al.*, 2014; Cianfanelli *et al.*, 2016)
		Hcp1 positively affects biofilm formation (opposite effect to Hcp2). Hcp3 positively regulates biofilm formation (in the presence of Hcp1 and Hcp2)	(Wang *et al.*, 2018)
		Negative effect of Hcp1 on the bacterial motility and protease production. Increased bacterial mortality and protease production.	(Sha *et al.*, 2013)

that T3SS and T3SS effector proteins/toxins have a bidirectional influence and contribute to destabilisation of the cell cytoskeleton, causing disturbance of normal physiological functions (such as preservation of cellular architecture, vesicular transport, phagocytosis). Simultaneously, this leads to deactivation of the host alarm system recognizing infection and inducing immune response. A predominant role of the complex interactions between T3SS effectors was highlighted in this activity.

The T3SS system is a sophisticated nano-syringe device which consists of ~20–25 different proteins and includes three main elements (Burkinshaw & Strynadka, 2014; Notti & Stebbins, 2016; Deng *et al.*, 2017; Wagner *et al.*, 2018; Pena *et al.*, 2019):

– Secretion apparatus – a structure through which protein toxins/effectors are delivered across the inner and outer membrane (Gaytan *et al.*, 2016; Deng *et al.*, 2017);

– Injection needle – a structure that facilitates bridging the gap between bacteria and the host cell, and transport of substances into the host (Park *et al.*, 2018; Lara-Tejero & Galan, 2019);

– Translocation apparatus – a structure by which effectors and toxins are translocated from the needle (Akopyan *et al.*, 2011; Mattei *et al.*, 2011).

A unique feature of the multiprotein T3SS system is the programmed secretion activity in some bacteria, which helps to avoid overproductive secretion of effectors (Gaytan *et al.*, 2016). The mechanisms for T3SS have not yet been fully investigated. The secretion of effectors is considered to be hierarchical, as demonstrated by Lara-Tejero and others (Lara-Tejero *et al.*, 2011). Type III secreted proteins are targeted to the secretion apparatus *via* a secretion signal located within the first 20 N-terminal amino acids. The secretion signal is poorly preserved at the primary amino-acid sequence level, although it shows some specific characteristics, e.g. enrichment in serine, threonine, isoleucine, and proline. Additionally, customized chaperones that bind a ~100-amino acid domain placed immediately downstream of the amino-terminal secretion signal for substrate targeting are necessary. In a partially unfolded state retaining its secondary structure, this domain is held by binding to the chaperone. The secondary structure configuration is an additional targeting signal, determines the location of the bound substrate in the secretion hierarchy, and primes the substrates for secretion (Galan & Waksman, 2018).

T6SS SECRETION SYSTEM AND EFFECTOR PROTEINS

Another secretion system that has been identified in *Aeromonas* is T6SS, i.e. the so-called Vas (virulence-associated secretion). T6SS, like T3SS, is a Sec independent system with an ability to transport protein effectors (Lien & Lai, 2017) directly to the cell surface or the host cell (Pukatzki *et al.*, 2009; Trunk *et al.*, 2018; Lewis *et al.*, 2019; Fernandez-Bravo & Figueras, 2020). The effector valine-glycine repeat G proteins (VgrG) and hemolysin co-regulated protein (Hcp) are the best known among the secreted ones (Whitney *et al.*, 2014). Translocation of T6SS-secreted Hcp in human colonic epithelial cells infected with *A. dhakensis* has been investigated by Suarez et al. Translocation has been shown to lead to apoptosis of host cells following activation of caspase 3 (Suarez *et al.*, 2008). Other research has indicated an inhibitory effect of Hcp on bacterial phagocytosis (Suarez *et al.*, 2010). In another paper, cytotoxic effects of VgrG1

on host cells *via* ADP-ribosylation of actin were demonstrated (Suarez *et al.*, 2010).

The type VI secretion apparatus is a versatile molecular machine consisting of two parts, namely a syringe-like structure extending to the cell membrane and a membrane-bound protein complex (Ho *et al.*, 2014). Recent studies have shown that the syringe-like structure is structurally analogous to the bacteriophage systolic tail (Zoued *et al.*, 2014; Basler, 2015), although it is much longer than the systolic tail (Veesler & Cambillau, 2011; Uchida *et al.*, 2014). It is assumed that the syringe-like structure includes the VgrG, Hcp, and VipA/VipB proteins (Uchida *et al.*, 2014). Sha and others (Sha *et al.*, 2013) evaluated the role of T6SS effector proteins co-regulated by hemolysin (Hcp) and valine-glycine repeat G proteins (VgrG: VgrG-1, -2 and -3) in *Aeromonas hydrophila* pathogenesis. Besides their predicted role as structural components and effector proteins, the experimental data clearly indicated that Hcp and VgrG paralogs also affected bacterial motility, protease production, and biofilm formation. The results showed that the Hcp and VgrG paralogs found in the T6SS cluster were largely involved in formation of the T6SS structures, while Hcp-1 and VgrG-1 located outside the T6SS cluster were T6SS effectors. Considering the influence on bacterial physiology, Hcp-1 exerted an effect on bacterial motility and production of a protease; in its absence, an increase in both types of activity was noticed. Similarly, VgrG-1 has been found to play an important role in regulating bacterial protease production, while VgrG-2 and VgrG-3 were crucial in regulating bacterial motility and biofilm formation. The contribution of two T6SS effectors of *Aeromonas hydrophila* to their virulence and the function of T6SS in both *in vitro* and *in vivo* models of infection have been established (Sha *et al.*, 2013). Recent analysis of the *hcp1* and *vgrG1* genes from T6SS has demonstrated that their deletion from a virulent *A. hydrophila* isolate leads to reduction of their virulence (approx. 2-fold) in relation to the parent strain (Tekedar *et al.*, 2018). This proves a significant contribution of these genes in the *Aeromonas* virulence potential. The list of T3SS, T2SS, and T6SS effector proteins with defined biochemical activities is included in Table 1.

OUTER MEMBRANE VESICLES: SECRETION SYSTEM TYPE 0

In the last few years, a new secretion system called type 0 has been described in Gram-negative bacteria. This system releases molecules inside particles derived from the outer membrane, called the outer membrane vesicles (OMVs), into the extracellular environment (Jan, 2017; Shehata *et al.*, 2019). OMVs are spherical nanoparticles with a diameter in a range of 50 to 250 nm. They are formed by the lipid bilayer, phospholipids, and outer membrane proteins. The vesicles arise when a protuberance develops in the membrane, which is eventually released as a vesicle. Several functions of OMVs have been described, including DNA transport (Avila-Calderon *et al.*, 2018). Although OMVs have been studied extensively in bacterial pathogens, the data on their composition are still incomplete. Avila-Calderón et al. performed a proteomic analysis to determine the composition of purified OMVs from *A. hydrophila* ATCC® 7966™ and their effect on host cells. The authors found 211 unique proteins in OMVs from *A. hydrophila*, among which the HcpA protein, RtxA toxin, and haemolysin Ahh1 are well-known determinants of virulence. It has been also shown that OMVs induced activation of lymphocyte and monocyte apoptosis. Over-expression of pro-inflammatory cytokines was also demonstrated (Avila-Calderon *et al.*, 2018). Due to their high immunogenicity, OMVs have been successfully applied as a vaccine platform against sepsis and bacterial meningitis. The use of Gram-negative OMVs as a vaccine platform is facilitated by engineering heterologous antigens to the vesicles. Since antigens retain a native conformation and are able to target a specific immune response, addition of heterologous proteins to OMVs has become a very promising strategy in the field of bioengineering of bacterial outer membrane vesicles as a vaccine platform (Gerritzen *et al.*, 2017).

CONCLUSION

Pathogenesis of *Aeromonas* infections is regarded to be multifactorial. Knowledge about the contribution of virulence determinants, including extracellular hemolysins, aerolysin, and effector proteins/toxins secreted *via* secretion nanomachines, is crucial and provides valuable insights into pathogenic mechanisms. The presence of factor-encoding and regulatory genes which can modulate bacterial virulence is associated with high variability between strains and species. Gene expression within *Aeromonas* species can be also differentiated, depending on the environmental conditions, such as the human host or water. Protein secretion systems are critical to bacterial virulence and interactions with other organisms. *Aeromonas* utilize various secretion machines, e.g. two-step T2SS system, for secretion of bacterial toxins and peptidases, including GCAT and aerolysin. Unlike T2SS, T3SS enables one-step secretion and translocation of microbial toxins or effector proteins with diverse biochemical activities into the host cells, causing disruption of the actin cytoskeleton, induction of apoptosis, signal transduction prevention and phagocytosis. T3SS, as the common virulence mechanism in *Aeromonas*, is one of the widely studied bacterial virulence determinants and its importance in the bacteria-host interactions is unquestioned. Among other weapons, T6SS is found in *Aeromonas* sp., which is used for both – the transport of microbial toxins into the host cell and secretion of some bacterial toxins, promoting bacterial dissemination. What is important, both the T3- and T6-secretion systems operate independently, which significantly affects the bacterial virulence mechanisms. Although various factors have been attributed to bacterial virulence, several others still remain to be discovered (Reynolds, 2009; Khajanchi *et al.*, 2010; Rangel *et al.*, 2019; Reyes-Rodriguez *et al.*, 2019). Recently, a growing number of scientific reports have suggested that environmental factors have a significant impact on the evolution of new metabolic adaptations that may be associated with bacterial pathogenicity (Staib & Fuchs, 2014).

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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