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Short Communication

Bioinformatics on Unproven Secretion Systems of *Vibrio Parahaemolyticus* -

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ABSTRACT

Several investigations were made in recent years related to Type 3 Secretion System (T3SS) and Type 6 Secretion System (T6SS) of *Vibrio parahaemolyticus*. However, numerous pathogenicity islands in genomes of *V. parahaemolyticus* remain unexploited. Considering the presence of other secretion systems such as T1SS, T2SS, T4SS and T5SS, which are associated with the pathogenicity in other bacteria exploring the Whole Genome Sequence (WGS) data, we identified the presence of T1SS-specific *Escherichia coli* HlyA, and *Morganella morganii* Vibrio ferrin homologs in *V. parahaemolyticus*. The T2SS encodes tight adherence (tad) locus for bacterial adherence and production of biofilm in *Actinobacillus actinomycetemcomitans*. This gene locus was identified as pilin in *V. parahaemolyticus*. Presence of VCA0120 and csu, the pilus encoding gene homologs in the environmental *V. parahaemolyticus* strains supports the view that part of T4SS may be active in this *Vibrio*. This information should be explored further in the diseases progress using *In vitro* and *In vivo* models.

Keywords: *Vibrio parahaemolyticus*; Secretion systems; Gene cluster comparison

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative, aquatic halophilic bacterium that inhabits in estuarine and marine environments. It causes three major diseases in humans; gastroenteritis, wound infections and septicemia. Among these, gastroenteritis is the most common with clinical symptoms of acute diarrhoea, abdominal cramps, nausea, vomiting, headache, and low-grade fever [1]. These symptoms are associated with seafood-borne infections, caused by *V. parahaemolyticus*, mostly in Southeast Asia. Based on the somatic (O) and capsular (K) antigens, several serotypes of *V. parahaemolyticus* have been identified. A unique serotype O3:K6 of *V. parahaemolyticus* has emerged in India during 1996 and spread to several countries. The other serotypes, such as O4:K68, O1: K25 and O1: KUT (untypeable) that subsequently appeared had similar virulence gene carriage and other molecular characteristics identical to that of the O3:K6 serotype. Pulsed-Field Gel Electrophoresis (PFGE), Variable Number Tandem Repeat (VNTR) and Multilocus Sequence Typing (MLST) of these serotypes displayed close genetic relationship with the pandemic O3:K6 serotype [1-3]. The clinical isolates of *V. parahaemolyticus* produce either the Thermostable-Direct Hemolysin (TDH) or TDH-Related Hemolysin (TRH) that are encoded in the *tdh* and *trh* genes, respectively [4]. The environmental strains on the other hand rarely have these virulence genes [5,6]. Strains devoid of any of these virulence markers are known to produce cytotoxicity and enterotoxigenicity *In vitro*. This suggests that other unknown virulence factors may have a role in the pathogenicity of *V. Parahaemolyticus* [7]. *V. parahaemolyticus* is well adapted for both natural and host environments with the ability to respond to rapid changes in the extracellular milieu [8]. Intestinal colonization of this bacterium is essential for the disease progress. Once the pathogen enters into the intestine, it induces extensive modification of intestinal microvilli by rearranging the cytoskeleton in host cells. This can lead to electrolyte imbalance and malabsorption of nutrients, which eventually cause secretory diarrhoea [9]. Using adult murine model, *Vibrio*-specific regulatory system ToxRS was shown to be important for colonization and survival under different stress conditions. Recently, Extra-Cytoplasmic Function (ECF) sigma factor, RpoE was identified as key cell envelope regulator for intestinal survival and colonization of vibrios [8]. During the course of infection, bacteria interact with their host and manipulate host cell response by secreting different types of protein. These proteins are located on the surface of bacteria that can be delivered into the host cells. To secrete exoproteins, bacteria utilize different machineries, which are called secretory system. Until date, six different types of secretion systems have been identified in Gram-negative bacteria, from type 1 (T1SS) to Type 6 Secretion Systems (T6SS) [10]. T3SS and T6SS are well studied and found to

be associated with the pathogenesis of *V. parahaemolyticus*. In this article, we have shown the linkage of unreported secretion systems in *V. parahaemolyticus* with other pathogens by a comparative analysis with submitted gene sequences to the National Center for Biotechnology (NCBI).

METHODOLOGY

Secretion systems associated proteins of (gene products) pathogens and their corresponding genes related data were from open source databases such as PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), PubMed Central (PMC) - (<http://www.ncbi.nlm.nih.gov/pmc>) and other open access journals available in the World Wide Web. The protein sequences of genome sequenced clinical *V. parahaemolyticus* strain RIMD 2210633 and environmental strain FDA - R31 were obtained from public domain (<https://www.ncbi.nlm.nih.gov/genome>). The sequence data collected from above were analysed using DNASTAR (Madison, WI, USA), Clone manager and BLAST (PubMed) search programme.

RESULTS AND DISCUSSION

Type 1 Secretion System (T1SS)

V. parahaemolyticus colonizes in the intestinal tract and invades into enterocytes. This invasion is determined by the genes encoded in the T1SS and T3SS. In many bacteria, the gene clusters responsible for T1SS expression is located in either the chromosome or plasmids. The RTX (repeat in toxin) toxin family is a group of cytotoxins secreted by Gram-negative bacteria into extracellular via T1SS. Proteins encoding the T1SS are diverse in nature but secreted by a common mechanism [11]. Hemolysin is the prototype of the RTX exotoxin family [12]. A well-studied hemolysin is the hlyA of uropathogenic *Escherichia coli* [13]. The structure of *E. coli* hemolysin T1SS consists of three components viz. HlyB, HlyD, and TolC [14]. These components are compared with RTX produced by vibrios such as *V. cholerae* and *V. vulnificus* and found to vary in the operon arrangements with the similarity ranges from 39 to 63% [15]. In *V. parahaemolyticus*, T1SS gene clusters were identified in the chromosome that encodes transport substrates, export functions and genes that promote transport substrates. Based on the Whole Genome Sequence (WGS) analysis obtained from the public domain, we detected that the *V. parahaemolyticus* strain FDA - R31 has a complete locus of *E. coli* hemolysin T1SS in the small chromosome with the variation in the protein sequence from 80 to 93% (Figure 1). The Outer Membrane Protein (OMP) of TolC, a component of T1SS is a multifunctional protein involved in many cellular processes and most important in the multidrug efflux pump system in *E. coli* and other bacteria. Among these, Resistance Nodulation cell Division



(RND) - type efflux transporters plays major role in the resistance to various antimicrobial agents and also virulence in many Gram-negative bacteria [16]. In *V. parahaemolyticus*, multiple and active RND - type efflux transporters has been identified in the chromosome [17]. Phylogenetic analysis of components of RND - type efflux transporters in *V. parahaemolyticus*, *V. cholerae*, *V. fischeri*, *V. harveyi* and *V. vulnificus* showed the presence of VmeCD along with the other multidrug efflux transporters [17]. Multiple deletions in these efflux encoding genes have shown significant increase in the sensitivity of *V. parahaemolyticus* towards many antimicrobial agents including bile acid and decrease in fluid accumulation in rabbit ileal loops [17].

V. parahaemolyticus utilizes iron through their siderophore. The secreted protein Vibrioferriin, which binds with heme to form ferric vibrioferriin complex on the outer membrane, which drives iron for *In vivo* survival and proliferation of bacteria [18]. Vibrioferriin has 16% similarity with hemophore HasA (Table 1), which is T1SS substrate of *Serratia marescens* [19]. A comparative study of the heme iron utilization systems of pathogenic vibrios such as *V. cholerae*, *V. alginolyticus* and *V. parahaemolyticus* showed that all had similar genetic constitution [20]. At the functional level, *V. parahaemolyticus* had identical amino acid sequence comparable to that of *V. cholera* [21]. Ferric Vibrioferriin receptor encoding gene *pvuA*-disrupt mutant study show decreased utilization of Ferric Vibrioferriin and this gene had 48% similarity with RumA of ferric rhizoferrin receptor of *Morganellamorganii* [22]. The IutA homologue of *V. parahaemolyticus* encodes an outer-membrane protein, which functions as the receptor for ferric aerobactin sharing 43% genetic homology with the *E. coli* IutA protein. Hybridization assay revealed that these homologs are widely distributed in clinical and environmental isolates of *V. Parahaemolyticus* [23]. *V. parahaemolyticus* utilize enterobactin via *PeuA* receptor under iron-limiting conditions [24]. These findings supported that *V. parahaemolyticus* uptake heme via siderophore-dependent uptake mechanism specific to surface receptors, activated through the trans envelope, TonB-ExbB/ExbD complex than Heme acquisition system (Has). These are heme uptake routes in bacteria mediated by the T1SSs.

Type 2 Secretion System (T2SS)

T2SS is one of the most flexible systems used by Gram-negative bacteria for transport of diverse proteins including hydrolytic enzymes and virulence factors from the periplasm across the outer membrane into the extracellular space [25]. This system involves a set of 12 - 15 different proteins, many of them in multiple copies located in the

Table 1: List of unreported secretion system homologs in *V. parahaemolyticus* compared to other bacteria.

Secretion system	Analogous proteins in <i>V. parahaemolyticus</i>	Known functions in bacteria (Information retrieved from PubMed)
T1SS	1. Vibrioferriin protein has 16% similarity with hemophore HasA of <i>S.marescens</i> 2. <i>HlyA</i> hemolysin transport protein similar to <i>E. coli</i> with homology between 80 and 93%	1. Nutrient acquisition - (iron scavenger protein, HasA) 2. Virulence (hemolysins) 3. Antibiotic resistance (RND pumps)
T2SS	1. Extracellular Protein Secretion (Eps) of <i>V. Parahaemolyticus</i> has 56% homology with <i>V. Cholerae</i> Eps protein. 2. Tight adherence (Tad) proteins act as a pseudopilins in <i>V. Parahaemolyticus</i> , which are similar to Tad proteins of <i>A. actinomycetemcomitans</i>	1. Transports folded proteins from periplasm into the extracellular environment via pseudopilus, Sec or Tat system. Substrates e.g., Hydrolysing enzymes
T4SS	A similar protein related to <i>IcmF</i> of <i>V. cholerae</i> and <i>L. pneumophila</i> . <i>IcmF</i> is responsible for motility, conjugation and adherence to Epithelial cells.	1. Mediate the conjugation. 2. Secretion transforming protein e.g. <i>H. pylori</i> . 3. Toxin production e.g. <i>Bordetella pertussis</i> . 4. Secretes protein to Support an intracellular lifestyle of bacteria e.g. <i>L. pneumophila</i> .
T5SS	Patatin-like lipolytic enzyme present in <i>V. parahaemolyticus</i> . This protein shared 32% homology with same protein of <i>P. aeruginosa</i> .	1. Cell to cell adhesion 2. Biofilm formation

complex structure of inner and outer membranes. In *V. cholerae*, this system is called Extra Cellular Protein (ECP) and in Enterotoxigenic *E. coli* (ETEC) and other bacteria this helps in the General Secretory Pathway (GSP) that spans from a regulatory ATPase in the cytoplasm to the actual pore in the outer membrane [25]. The structure of T2SS consists of the outer membrane complex, the pseudopilus, and the inner membrane platform. The outer membrane complex composed of EpsD, the pseudopilus consists of five different pseudopilins such as EpsG to EpsK. The inner membrane platform consists of bitopic membrane proteins EpsL and EpsM; the membrane-anchored EpsC; the integral membrane protein EpsF; and the membrane-associated “secretion ATPase” EpsE in the cytosol [26]. Periplasmic domain of EpsL (peri-EpsL) in *V. parahaemolyticus* shared 56% homology with *V. cholerae* and this involves in the circular permutation of ferredoxin along with the other sub-domains [25].

It was shown that the secretion system associated Tad (tight adherence) loci are essential for biosynthesis of long and bundled Flp pili, which support the bacterial adherence and biofilm formation [27,28]. The biosynthesis of Flp pilus involved in 12 - 13 genes of *tad* locus, in *Actinobacillus actinomycetemcomitans*, which share similarity to other bacteria having T2SSs, type IV pilus systems and few genes with T4SS NTPases [29]. The *tad* locus protein TadV functions as prepilin peptidase in maturation of the Flp pilin. In *A. actinomycetemcomitans*, the pre-TadE and pre-TadF inner membrane proteins are responsible for adherence and biofilm formation [29].

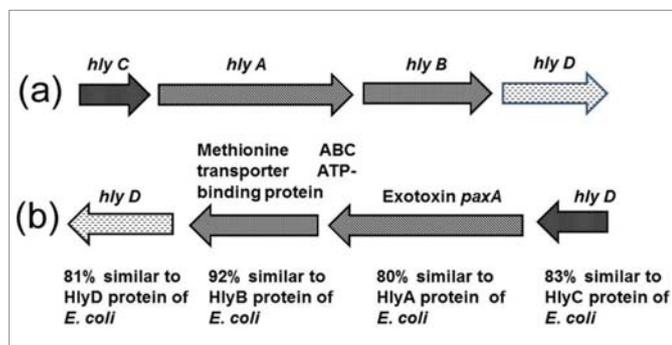


Figure 1: Comparison of *hly* gene clusters of *V. parahaemolyticus* and *E. coli*. (a) *hly* genes cluster of *E. coli* obtained from accession number M10133. (b) Similar genes cluster of *V. parahaemolyticus* obtained from accession number CP006005.

In *A. actinomycetemcomitans* and *V. parahaemolyticus*, these highly conserved Tad proteins act as pseudopilins [29]. *V. parahaemolyticus* strain RIMD 2210633 has the entire Tad locus as reported in *A. actinomycetemcomitans* and found vary at the amino acid level (Figure 2). However, experimental evidences are needed to show the importance of Tad locus in the pathogenesis of *V. parahaemolyticus*.

Type 4 Secretion System (T4SS)

T4SSs is a multiprotein complex that can transport DNA, toxins and effector proteins through bacterial membranes to the extracellular milieu or directly into the cytoplasm of other cells [30]. In several bacteria, T4SS system has been established. *Legionella pneumophila* had shown to survive in the host cell by injecting a vast number of toxins through its Dot/Icm type IVB Secretion System (T4SS). T4SS effector proteins are involved three classes such as translocation channel, the pilus and ATPase proteins [31]. The T4SS effector proteins such as IcmF and IcmH (DotU) of *L. pneumophila* were non-essential components of this system and required for cytotoxicity of *L. pneumophila* toward mammalian and Dictyostelium discoideum cells [32,33] and also shared significant sequence similarity to plasmid genes involved in conjugation [34].

Protein encoding genes of *icmF* and *icmH* (*dotU*) shared sequence similarity with *vasK* (VCA010) and *vasF* (VCA0115) of *V. cholerae*. These genes are involved in regulating the motility of the pathogen and adherence to the intestinal epithelial cell line [35]. Deletion mutant of these genes in Dictyostelium sp. model has been reported as attenuation of virulence [33]. Even though the T4SS has not been recognized in *V. parahaemolyticus*, we identified VCA0120 protein of *V. cholerae* that had 52% protein sequence homology with recently sequenced Canadian *V. parahaemolyticus* strains [36]. This has been annotated as T6SS protein in *V. parahaemolyticus*, accession no. KHF17383 [36], which had 23% similarity with *V. parahaemolyticus* strain RIMD 2210633 locus VP1408.

Pilus is one of the sub-groups of T4SS, which is responsible for transfer of proteins to establish cell to cell contact [37]. Pilus has been recognized as one of the adherent factors of pathogenic bacteria [38]. The pili identified in different *V. parahaemolyticus* strains has the adhesive property in rabbit intestine [39,40]. In *Acinetobacter baumannii*, the *csu* operon was responsible for attachment and biofilms formation and this enable bacteria to persist in their natural environments or in host tissue [41]. Interestingly, a similar locus has been found in clinical and environmental strains of *V. Parahaemolyticus* [41,42]. *V. parahaemolyticus* utilizes IV-A pilus genes as sophisticated machinery for survival in natural environments. In *V. parahaemolyticus* RIMD 2210633, two sets of type IV-A pilus have been identified, one is chitin-regulated pilus; ChiRP, which is similar to *P. aeruginosa* and *Vibrio* spp. and the

other, is similar To Mannose-Sensitive Hemagglutinin (MSHA). These two genes contribute in the biofilm formation in different pathways [43]. MSHA pilus was identified as a significant factor in bacterial-host cell adherence and pathogenic to Caco-2 cells. MSHA pilus act on glycans receptors such as asialo-GM1 ganglioside, lacto-N-fucopentaose I and lacto-N-difucohexaose I in the gastrointestinal tract and enable efficient colonization of the intestinal epithelium by *V. Parahaemolyticus* [44]. Type IV pili was shown to contribute persistence of *V. parahaemolyticus* in Pacific oysters [45].

Type 5 Secretion System (T5SS)

A protein secreted by T5SS is known as autotransporters, which comprised of autotransporter family (classical type), the Two-Partner System (TPS) and the Oligomeric Coiled coils adhesion (Oca) family. The autotransporter secretion process involves first the translocation of the precursor through the inner membrane and then its translocation through the outer membrane via a pore formed by a β -barrel [46]. TPS are translated by TpsA and TpsB proteins. TpsA is exoproteintranslocated through the outer membrane via β -barrel pore formed by TpsB [46]. The auto transporter of Oca family was identified in *Yersinia enterocolitica* and adhesion protein involved in this process is YadA, which is subfamily of surface-attached oligomeric auto transporters [47]. Patatin-Like Protein (PlpD) has been described in *P. aeruginosa*, which shared the common features of classical type of auto transporter and TpsB of TPS in one polypeptide chain [48]. More than 200 PlpD orthologues exist among pathogenic and environmental bacteria, which could secrete numerous PLPs in bacteria through this system [49].

In our analysis, we found that Patatin-like protein shared 32% homology with hypothetical protein of gene locus VP2496 of *V. parahaemolyticus* strain RIMD 2210633. However, this region has been annotated as serine protease in genome sequenced *V. parahaemolyticus* strains. As a part of T5SS, two adhesions intimin and invasin have been described in *E. coli* and *Yersinia* spp., respectively, but these proteins did not have any similarity in the *V. parahaemolyticus* genome.

PERSPECTIVE

Our study suggests that *V. parahaemolyticus* has other important secretion systems namely T1SS, T2SS, T4SS and T5SS, in addition to the known T3SS and T6SS, which are homologous to Gram-negative bacteria. Vibrioferrin, *HlyA*, *Eps*, *Tad*, pseudopilins and patatin-like lipolytic enzyme are the important secretion components found to have the virulence of *V. parahaemolyticus*. Understanding of these secretion systems through novel *In vitro* and *In vivo* models could create new avenues for exploration of pathogenesis and effective infection control.

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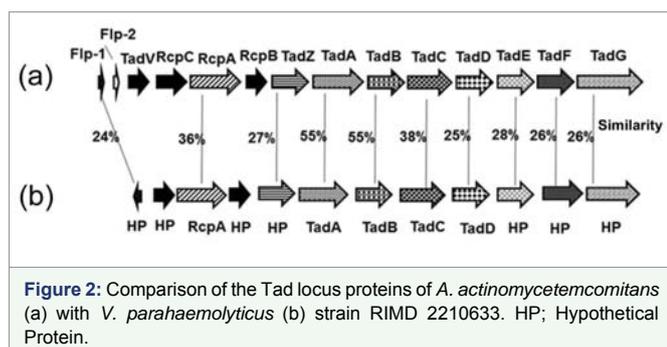


Figure 2: Comparison of the Tad locus proteins of *A. actinomycetemcomitans* (a) with *V. parahaemolyticus* (b) strain RIMD 2210633. HP; Hypothetical Protein.



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