

1 **Acetylome Profiling of *Vibrio alginolyticus* Reveals Its** 2 **Role in Bacterial Virulence**

3 Huanying Pang^{a,b,c,d,1}, Wanxin Li^{f,1}, Weijie Zhang^{b,c}, Shihui Zhou^{b,c}, Rowena Hoare^e,

4 Sean J. Monaghan^e, Jichan Jian^{b,c,d,*}, Xiangmin Lin^{f*}

5 ^aShenzhen Institute of Guangdong Ocean University, Shenzhen 510000, China;

6 ^bFisheries College, Guangdong Ocean University, Zhanjiang 524025, China;

7 ^cGuangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for

8 Aquatic Economic Animals, Zhanjiang 524025, China; Guangdong Key Laboratory

9 of Control for Diseases of Aquatic Economic Animals, Zhanjiang 524025, China

10 ^dKey Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese

11 Academy of Sciences, Qingdao266071, China; Laboratory for Marine Biology and

12 Biotechnology, Qingdao National Laboratory for Marine Science and Technology,

13 Qingdao 266071, China

14 ^eInstitute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

15 ^fFujian Provincial Key Laboratory of Agroecological Processing and Safety

16 Monitoring (School of Life Sciences, Fujian Agriculture and Forestry University),

17 Fuzhou, China

18

19 * Corresponding author: Jichan Jian, Tel./fax: +86-759-2339319; Address: College of

20 Fishery, Guangdong Ocean University, No. 40 of East Jiefang Road, Xiashan District,

21 Zhanjiang, Guangdong Province, 524025, China; E-mail: jianjic@gmail.com;

1 Xiangmin Lin, Tel: +86059183769440; Address: Agroecological Institute, Fujian
2 Agriculture and Forestry University, Fuzhou 350002, Fujian, China; E-mail:
3 xiangmin@fafu.edu.cn.

4 1 These authors contributed to the work equally and should be regarded as co-first
5 authors.

6

7

1 **Abstract:** It is well known that lysine acetylation (Kace) modification is a common
2 post-translational modification (PTM) that plays an important role in multiple biological
3 and pathological functions in bacteria. However, few studies have focused on lysine
4 acetylation modification in aquatic pathogens to date. In this study, the acetylome
5 profiling of fish pathogen, *Vibrio alginolyticus* was investigated by combining affinity
6 enrichment with LC MS/MS. A total of 2883 acetylation modification sites on 1178
7 proteins in this pathogen were identified. The Kace modification of several selected
8 proteins were further validated by Co-immunoprecipitation combined with Western
9 blotting. Bioinformatics analysis showed that seven conserved motifs can be enriched
10 among Kace peptides, and many of them were significantly enriched in metabolic
11 processes such as biosynthesis of secondary metabolites, microbial metabolism in
12 diverse environments, and biosynthesis of amino acids, which was similar to data
13 previously published for *V. parahaemolyticus*. Moreover, we found at least 102
14 acetylation modified proteins predicted as virulence factors, which indicate the
15 important role of PTM on bacterial virulence. In general, our results provide a
16 promising starting point for further investigations of the biological role of lysine
17 acetylation on bacterial virulence in *V. alginolyticus*.

18 **Keywords:** *Vibrio alginolyticus*; Acetylome; Bacterial virulence; Proteomics

19

1. Introduction

With the development of high-resolution mass spectrometry and high affinity purification technology for lysine acetylated peptides, lysine acetylation (Kace) modification of proteins has been well validated to be a widely distributed post-translational modification (PTM) in both eukaryotes and bacteria [1, 2]. Moreover, this PTM was reported to play important roles in maintaining the physiological and pathologic function. For example, in mammalian cells, some Kace modified proteins such as HDAC6 and Tau, were found to be involved in human diseases such as inflammation and Alzheimer's disease [3, 4]. In bacterial cells, the distribution of Kace modification has been well documented in dozens of species, including *Vibrio parahaemolyticus*, *Escherichia coli* and *Mycobacterium tuberculosis* [5-7], and was reported to be involved in many basal physiological functions such as chemotaxis, virulence, and antibiotic resistance [8-10]. Due to the diverse nature of this PTM, it has been suggested that lysine acetylation could provide a new target for vaccine and drug development based on an understanding of the regulatory mechanisms of this modification [11, 12]. However, although there are more studies reporting the roles of Kace modification on diverse cellular functions, the profile and physiological functions of this PTM still remain elusive in many species, especially fish pathogens. Until now, few acetylome profiles in fish pathogens have been reported e.g. *V. parahaemolyticus* and *Aeromonas hydrophila* [5, 13]. Given the importance of infectious diseases in aquaculture, it is necessary to carry out large scale identification of this PTM and compare various Kace modification profiles in more aquatic pathogens.

1
2 *Vibrio alginolyticus* a well known fish pathogen in the aquaculture industry which can
3 infect orange-spotted grouper (*Epinephelus coioides*) [14], *Lutjanus*
4 *erythropterus*(Bloch) [15], and large yellow croaker (*Larimichthys crocea*) [16] fish.
5 Each year, the fish diseases caused by this pathogen leads to an economic loss of
6 approximately \$150 million. Recently, several proteins such as HopPmaJ [17], Acfa
7 [18] and VscO [19] have been reported to be involved in invasion of the host as a
8 virulence effector, as well as protein RpoS affect on adhesion to bacteria, growth,
9 hemolysis, biofilm production, movement [20], and SodB protein influence of bacterial
10 motility and adhesion, leading to changes in bacterial virulence [21]. However, the
11 intrinsic virulence mechanisms are still largely unknown, especially for the role of
12 Kace modification.

13
14 In this study, the acetylome profile of the pathogenic *V. alginolyticus* was performed by
15 combining high-accuracy MS and a highly specific Kace affinity method, and results
16 were compared with previously published *V. parahaemolyticus* acetylome data.
17 Bioinformatic analysis of the resulting proteins revealed that these Kace proteins are
18 involved in various biological processes and central metabolism pathways, which is
19 similar with that seen in *V. parahaemolyticus*. Moreover, we also found that several
20 virulence effectors, such as HemL, FabB, FabD, FabF-3 and PhoR, are acetylated,
21 which indicate PTM of these proteins may play an important role in bacterial-host
22 interactions and should be further investigated in the future. Our data enrich the

-
- 1 understanding of Kace modification profiling in prokaryotes and provides new insights
 - 2 into the potential role of PTMs in fish pathogens.
 - 3

1 **2. Materials and methods**

2 **2.1 Bacterial strains and Sample preparation**

3 *V. alginolyticus* strain HY9901, isolated from *Lutjanus erythropterus* [15], was used in
4 this study. A single colony was cultured overnight in Luria-Bertani (LB) broth at 28° C
5 on a shaker at 200 rpm, and then diluted to 1:100 in fresh LB medium. This was
6 cultured to logarithmic growth stage, the cells collected by centrifugation at 8000 rpm,
7 4° C for 10 min, and washed twice with cold PBS (phosphate buffer saline). The
8 pellets were resuspended with 8 M Urea and 0.2% SDS in 50 mM Tris-HCl (pH=8)
9 and then broken by ultrasonication in an ice bath for 5 min before centrifugation at
10 12000 g for 15 min at 4°C. The supernatant was collected and the protein
11 concentration was determined using the Bradford assay. The supernatant was
12 incubated with 2 mM DTT at 56°C for 1 hour followed with adding 20 mM
13 iodoacetamide (IAA) for 1 hour at room temperature in the dark as previously
14 described [22]. Four volumes of pre-chilled acetone were added to the supernatant to
15 precipitate protein at -20°C for 2 h and the precipitate was then washed twice with
16 cold acetone. The pellet was dissolved in 8 M urea in 0.1 M triethylammonium
17 bicarbonate (TEAB, pH=8.5) and the protein concentration was determined again
18 before further procedures.

20 **2.2. Immunoaffinity enrichment of lysine-acetylated peptides**

21 10 mg of protein was digested with Trypsin Gold (Promega) at 1:20 (Trypsin: protein =
22 1:20) ratio at 37°C for 16 h as previously described [23]. The digested peptide was

1 desalted with C18 cartridge (Waters Inc., Milford, MA, USA) and dried by a CentriVap
2 vacuum concentrator (Labconco Inc., Kansas City, MO, USA). Lyophilized peptides
3 were dissolved in MOPS IAP buffer (50 mM MOPS, 10 mM KH₂PO₄ and 50 mM NaCl,
4 pH 7), mixed with anti-acetyl-lysine beads (Cell Signaling Technology) for 2.5 h at 4°C,
5 and then washed twice with MOPS IAP buffer by centrifugation for 30 s at 3000 g at
6 4°C. The Kace enriched peptides were eluted with 0.15% TFA (Trifluoroacetic acid)
7 and then desalted using peptide desalting spin columns (Thermo Fisher) before MS
8 analysis.

9

10 **2.3. LC-MS/MS Analysis**

11 The desalted peptides were analyzed using an EASY-nLCTM 1200 UHPLC system
12 (ThermoFisher) coupled to an Orbitrap Q Exactive HF-X mass spectrometer
13 (ThermoFisher) in the data-dependent acquisition (DDA) mode [24]. Briefly, peptides
14 in 0.1% FA (Formic acid) were injected onto a Acclaim PepMap100 C18 Nano-Trap
15 column (2 cm×100 μm, 5 μm) and separated on a Reprosil-Pur 120 C18-AQ analytical
16 column (15 cm×150 μm, 1.9 μm) with a 120 min linear gradient from 5 to 100% buffer
17 B (0.1% FA in 80% ACN) in buffer A (0.1% FA in H₂O) at a flow rate of 600 nL/min. The
18 solvent gradient was set as follows: 5-10% B, 2 min; 10-40% B, 105 min; 40–50% B, 5
19 min; 50–90% B, 3 min; 90–100% B, 5 min.

20

21 For DDA, the Q-Exactive HF-X mass spectrometer was operated in positive mode
22 with spray voltage of 2.3 kV and the capillary temperature was 320°C. Full MS scans

1 from 350 to 1500 m/z were acquired at a resolution of 60000 (at 200 m/z) with an AGC
2 target value of 3×10^6 . From the full MS scan, a maximum number of 40 of the most
3 abundant precursor ions were selected for higher energy collisional dissociation (HCD)
4 fragment analysis at a resolution of 15000 (at 200 m/z), a maximum ion injection time
5 of 45 ms, a normalized collision energy of 27%, an intensity threshold of 8.3×10^3 , and
6 the dynamic exclusion parameter set at 60 s.

7

8 **2.4 Bioinformatics**

9 The Gene Ontology (GO) annotation and KEGG pathway were enriched by
10 Omicsbean online software (<http://www.omicsbean.cn/>) [25]. STRING software
11 (<https://string-db.org/cgi/input.pl?sessionId>) combined with Cytoscape were used to
12 predict protein-protein interaction network [26], and motif analysis was performed by
13 the Motif-X software. Virulence factors (VFs) retrieval was carried out using the
14 Virulence Factor Database (VFDB) database (<http://www.mgc.ac.cn/VFs/main.htm>),
15 which is a comprehensive public store and online platform for deciphering bacterial
16 pathogenesis. The VFs homologs were identified using Blast analysis and using
17 E-value $< 1.0^{-5}$ as cutoff [27].

18

19 **2.5 CO-IP and Western blot**

20 Specific polyclonal antibodies to D0X475 (Uncharacterized protein) and OmpH
21 (Chaperone protein Skp) were used to precipitate target proteins. *V.*
22 *alginolyticus* strain cell lysates (500 μ g) were interacted with D0X475 and OmpH

1 antibody at 4°C overnight. Protein A/G beads washed three times with PBS buffer
2 were added to the lysates at 4°C for 1-3 h [28]. The beads were pelleted at 4°C,
3 followed by five washes with PBS buffer. 50µL of loading sample buffer (250 mM
4 Tris-HCl PH=6.8, 10% SDS, 0.5% bromophenol blue 50% glycerol and 5%
5 β-mercaptoethanol) was added to the pellet, boiled for 5 min, and subsequently
6 analyzed by SDS-PAGE and western blot.

7

8 For western blot, proteins were run 12% 1-DE gels and transferred to a polyvinylidene
9 fluoride (PVDF) membrane. The membranes were blocked in TBS (Tris buffered
10 saline) containing 0.05% (v/v) Tween 20 with 5% (w/v) skim milk and incubated 1 h at
11 room temperature. The primary antibodies used in the western blot were anti-Kace
12 (1:1000), anti-D0X475 (1:4000) and anti-OmpH (1:4000) and incubated overnight at
13 4°C. Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG was used as the
14 secondary antibody at a 1:5000 dilution. Finally, the membrane was visualized using
15 the ECL system (Bio-Rad, Hercules, CA, USA), and recorded by the ChemiDoc™ MP
16 (Bio-Rad, Hercules, CA, USA) imaging system [29].

17

18 **3. Results and Discussion**

19 ***3.1 Quantitative proteomic profiling of lysine acetylation peptides in V.*** 20 ***alginolyticus***

21 In order to map lysine acetylation sites in *V. alginolyticus*, proteins were isolated from
22 exponentially growing cells. Acetylated peptides were generated through purification

1 and digestion of these protein samples, followed by affinity enrichment and
2 purification. Peptides were further separated and identified by EASY-nLC™ 1200
3 UHPLC system coupled to an Orbitrap Q Exactive™ HF-X mass spectrometer (**Figure**
4 **1A**). Finally, we performed the first largescale analysis of lysine acetylated proteins in
5 *V. alginolyticus*. The results yielded a total of 2883 peptides and 2537 acetylated sites
6 on 1178 proteins (Supplemental Table S1 and S2). As shown in **Figure 1B**, the mass
7 error of most acetylated peptides ranged from -5 to 5 ppm, indicating an expected
8 error control from the MS dataset. The mass of most acetylated proteins was in the
9 range of 10-60 kDa (**Figure 1C**). The length of most of the peptides ranged from 7-20
10 amino acids, and exhibited distinct abundance depending on their lengths (**Figure**
11 **1D**), well in agreement with the property of peptides and the preparation standard of
12 protein sample. Moreover, the numbers of modification sites for each protein is mainly
13 from 1 to 10, and more than 45.2% of proteins contained only one acetylation sites,
14 and the 22.7%, 11.9%, 6.1%, and 14.1% of proteins with two, three and four or more
15 modification sites, respectively (**Figure 1E**), which was in common with many other
16 studies [30, 31]. In this study we found that D0WZ79 (*VMC_24790*), the component of
17 the pyruvate dehydrogenase (PDH) complex as the key enzyme in the pyruvate cycle
18 has the highest number of acetylation sites (24 acetylation sites). A previous study
19 has showed that pyruvate cycle provides respiratory energy in bacteria, and protect
20 bacteria from stressful environments, which indicate the important role of this protein
21 on intracellular metabolic regulation [32]. In addition, there were many
22 translation-related PTM proteins on 50S (except for S12, S14 and S20) and 30S

1 (except for L7, L8, L26 and L32) ribosome subunits. The results indicated that
2 acetylation may be involved in bacterial protein translation. In addition, 30S ribosome
3 subunits D0WW34 (*rpoB*) and D0WW35 (*rpoC*) have 20 and 17 modified sites,
4 respectively, and a previous study reported that knockout of *rpoB* gene reduced the
5 *rpoB-rpoC* mRNA level by two-fold and may lead to cell defects [33].

6

7 **3.2 Functional annotation and enrichment of acetylated proteins in *V.*** 8 ***alginolyticus***

9 To better understand the role and distribution of the acetylated modification in *V.*
10 *alginolyticus*, the GO functional annotations, KEGG pathway analysis and protein
11 domains of acetylation-modified proteins were analyzed. The GO analysis was
12 performed to gain insight into the potential functional role. In total, 1178 of the
13 acetylated proteins were analyzed according to their biological process, cellular
14 component and molecular function using OmicsBean online software. In the biological
15 process (BP) category, many of acetylated proteins were significantly enriched in
16 metabolic process, especially in organic substance metabolic process and cellular
17 metabolic process (**Figure 2A**). Consistently, in the cellular component (CC) category,
18 most of the detected lysine-acetylated proteins were involved in the cell part, cell,
19 intracellular, cytoplasm and intracellular part (**Figure 2B**). The molecular function (MF)
20 category, mainly comprised ion binding, small molecule binding, nucleoside binding,
21 nucleoside phosphate binding, and anion binding (**Figure 2C**). The GO analysis
22 results are consistent with previous results that showed that most lysine acetylated

1 proteins are categorized as involved in metabolic and biosynthetic processes and
2 located in the cytoplasm [5, 10, 34].

3

4 To gain further insights into the processes regulated by the acetylation, we mapped
5 the acetylated proteins to KEGG pathways (**Figure 2D**). The results showed that the
6 acetylated proteins identified were assigned to several groups, including metabolism
7 pathways, biosynthesis of secondary metabolites, biosynthesis of antibiotics,
8 microbial metabolism in diverse environments, and biosynthesis of amino acids. In
9 general, lysine acetylation occurs on many enzymes involved in metabolism, and
10 these findings were consistent with previous studies in *V. parahaemolyticus* [5].

11

12 Proteins are composed of domains, which are the units of protein structure, function
13 and evolution. In this study, the protein domain enrichment analysis showed that
14 proteins with P-loop containing nucleoside triphosphate hydrolase, nucleic
15 acid-binding, OB-fold, NAD(P)-binding domain superfamily and NAD(P)-binding
16 domain have a higher tendency to be acetylated (**Figure 2E**). The domain P-loop
17 containing nucleoside triphosphate hydrolase has been shown to have a significant
18 role in different forms of signal transduction, intracellular trafficking, and cytoskeletal
19 re-organization [35]. Previous studies have reported that the NAD(P)-binding domain
20 superfamily and NAD(P)-binding domain are particularly well suited to regulate
21 transcriptional activity in response to changes in cellular redox balance [36]. Our
22 results indicate that Kace modification may be involved in complicated biological

1 processes including protein biosynthesis and nucleic acid-binding.

2

3 **3.3 Analysis of acetylated-lysine sequence motifs**

4 In this study, we used the Motif-X extractor web tool [37] (a software that was
5 designed to extract overrepresented patterns from any sequence data set) to
6 determine the sequence motifs from all of the acetylated peptides in *V. alginolyticus*.

7 The analysis of the frequency of amino acids sequences from -10 to +10 surrounding
8 the acetylated lysine. Seven conserved motifs that can be enriched among acetylated
9 peptides, such as EK^{ac}, AK^{ac}, LK^{ac}, K^{ac**}K, DK^{ac}, L*K^{ac****}R, GDK^{ac} are shown in

10 **Figure 3.** The results showed that the EK^{ac}, AK^{ac}, LK^{ac}, K^{ac**}K, DK^{ac} were significantly
11 preferred, suggesting that these conserved sites may be functionally important for
12 acetylation in *V. alginolyticus*. Similar results have been found in *Thermus*
13 *thermophilus* and *Mycobacterium tuberculosis* [7, 38]. LK^{ac}, EK^{ac} and DK^{ac} have also
14 been identified in the consensus motif, and LK^{ac} motif was most abundant in *Bacillus*
15 *amyloliquefaciens* [10], indicating that the acetylation sites of lysine are conserved.
16 Interestingly, it could be observed that the residue preferences for acetylated peptides
17 are -1 position, such as residue E, A, L and D, indicating the conservation of Kace
18 modification motifs.

19

20 **3.4 Protein-protein interaction network of the *V. alginolyticus* acetylome**

21 In order to investigate cell life activity involving acetylated proteins in *V. alginolyticus*,
22 the protein-protein interaction (PPI) network was established (**Figure 4**). On the basis

1 of STRING database analysis, we found that 36 ribosomal subunit proteins were
2 highly interconnected, indicating that the translation process may be regulated by
3 acetylation in *V. alginolyticus*, in accordance with *V. parahaemolyticus* and *M.*
4 *tuberculosis* results [5, 7]. Interestingly, a large number of ribosomal subunit proteins
5 were also succinylated in *A. hydrophila*, suggesting that ribosomal proteins have
6 “cross-talk” phenomenon [22]. Furthermore, most of the acetylated proteins were
7 involved in metabolic pathways, including the microbial metabolism in diverse
8 environments, carbon metabolism, biosynthesis of amino acids, and aminoacyl-tRNA
9 biosynthesis. These PPI networks, suggest that protein acetylation is a key PTM in *V.*
10 *alginolyticus* that contributes to cooperation and coordination of metabolic pathways.

11

12 **3.5 Validation of D0X475 and OmpH lysine-acetylated proteins using**
13 **Co-Immunoprecipitation and Western blotting.**

14 To further validate the identified lysine-acetylated results, 2 Kace proteins (D0X475
15 and OmpH) were selected and analyzed by Co-IP and Western blotting. The D0X475
16 and OmpH proteins were captured by their respective antibodies and then Western
17 blotting was performed with anti-acetylation and anti-target protein antibody,
18 respectively (**Figure 5**). The results showed that D0X475 and OmpH proteins
19 exhibited acetylation modifications consistent with lysine-acetylated proteomic data,
20 further validating our proteomics results.

21

22 **3.6 Comparison of lysine acetylome between *V. alginolyticus* and *V.***

1 ***parahaemolyticus***

2 *V. alginolyticus* and *V. parahaemolyticus* are both common aquatic microorganisms in
3 the marine environment with considerably high homology. A previous study reported
4 the lysine acetylome in *V. parahaemolyticus* and found 1413 acetylation sites on 656
5 proteins [5]. In this study, we compared the acetylated modified proteins in both
6 pathogens. Results showed that 408 proteins have orthologs in the acetylome of *V.*
7 *parahaemolyticus* (**Figure 6A**), indicating that they have considerable similarities in
8 functions such as signal transduction, energy metabolism, cytoskeleton and
9 transcription factor activity, to name a few. Furthermore, we analyzed the KEGG
10 pathway of the overlap acetylated proteins. The results showed that the common
11 pathways were related to biosynthesis of secondary metabolites, antibiotics, and
12 amino acid, microbial metabolism in diverse environments, carbon metabolites, and
13 ribosome. We also aligned *V. alginolyticus* acetylated peptides with *V.*
14 *parahaemolyticus* and found 372 acetylated peptides conserved (**Figure 6B**). Further,
15 the overlapping acetylated peptides for motif analysis, and the results showed that
16 two conserved motifs were enriched, such as K^{ac}**E and D**K^{ac}, indicating that there
17 are many conservation acetylation modification sequences in *Vibrio. spp.*

18
19 **3.7 Virulence factors of acetylated proteins in *V. alginolyticus***

20 To date, a number of acetylated proteins were reported to be involved in virulence in
21 various bacterial species, such as *Acinetobacter baumannii*, *Pseudomonas*
22 *aeruginosa*, *Staphylococcus aureus* and *V. parahaemolyticus* [5, 39-41]. Virulence

1 factors (VFs) of *V. alginolyticus* were retrieved from the VFDB database in order to
2 better understand the effect of Kace modification on *V. alginolyticus* virulence. A total
3 of 102 proteins were detected, accounting for 8.66% of the total proteins
4 (Supplementary Table S3). Further, analysis of the protein-protein interaction network
5 of VFs revealed that 63 proteins were found to interact (**Figure 7**). The LysR-family
6 transcriptional regulators are among the most widespread regulators in prokaryotes,
7 and control a wide range of biological processes in order to achieve optimal bacterial
8 survival and adaptation in adverse environments [42, 43], such as some LysR-family
9 regulators, RovMin in *Yersinia pseudotuberculosis*, OxyR in *Klebsiella pneumoniae*,
10 BexR in *Pseudomonas aeruginosa* have been shown to be important for regulating
11 virulence [44-46]. In this study N646_4035 had the most acetylation sites, and
12 sequence analysis revealed that it belongs to the LysR family transcriptional regulator,
13 which may be involved in the virulence of *V. alginolyticus*. In addition, N646_2304 and
14 N646_2853 two GGDEF family proteins were acetylated, and studies in *Xanthomonas*
15 *oryzae* reported that GGDEF-domain proteins are connected with virulence and
16 motility [47, 48], indicating that N646_2304 and N646_2853 may affect the virulence
17 of *V. alginolyticus*. However, it remains unknown how acetylation regulates virulence
18 in *V. alginolyticus* and that warrants further study in the future.

19

1 **4. Conclusion**

2 It is well known that lysine acetylation (Kace) modification plays an important role in
3 the multiple biological functions in bacteria whereas few reports focus on the lysine
4 acetylation modification profiling in aquatic pathogens such as *V. alginolyticus*. In this
5 study, using high-resolution mass spectrometry with a high affinity enrichment method,
6 we successfully identified a total a total of 2883 acetylation modification sites on 1178
7 proteins in *V. alginolyticus*. Our results showed that the many of Kace proteins
8 detected are involved in various metabolic pathways and translation processes, which
9 is similar with other bacterial species, especially *V. parahaemolyticus*. Moreover, we
10 also found a considerable number of Kace proteins were predicted as virulence
11 factors and may play important roles in bacterial virulence, potentially providing
12 targets for vaccine development. The virulence mechanisms and pathogen-host
13 interactions of this important aquaculture pathogen should be further investigated.

14 **Acknowledgments**

15 This work was funded by Shenzhen Science and Technology Project
16 (JCYJ20170818111629778), the National Key Research and Development Program
17 of China (2018YFD0900501), National Natural Science Foundation of China (No.
18 31402344, 31670129), Natural Science Foundation of Guangdong Province (No.
19 2017A030313174), Program for Innovative Research Team in Fujian Agricultural and
20 Forestry University (No.712018009), Innovation and Entrepreneurship Training
21 Program for College Students (No.CXXL2019041).

1 **Legends and Figures**

2 **Figure 1. Outline of *V. alginolyticus* lysine acetylation proteomics.** (A) Workflow
3 of quantitative proteomics analysis of lysine acetylation in *V. alginolyticus*. (B)
4 Distributions of mass errors for all identified lysine-acetylated peptides. (C)
5 Distribution of protein mass in the acetylated proteins. (D) Distribution of acetylated
6 peptides based on their length. (E) Distribution of acetylated sites in the acetylated
7 proteins.

8
9 **Figure 2. Functional classification of acetylated proteins in *V. alginolyticus*.**
10 (A-C) Biological Processes, Cell Components and Molecular Function enrichment
11 analysis of the acetylated proteins. (D) KEGG pathway-based enrichment analysis of
12 the acetylated proteins. (E) Domains enrichment analysis of the acetylated proteins.

13
14 **Figure 3. Properties of the acetylated peptides.** (A) Acetylation motifs and
15 conservation of acetylation sites. The height of each letter corresponds to the
16 frequency of that amino acid residue in that position. The central K refers to the
17 acetylated lysine. (B) Number of peptides containing significantly enriched motifs as
18 identified using MotifX.

19
20 **Figure 4. Protein-protein interaction network of the *V. alginolyticus* acetylome.**
21 Protein-protein interaction network obtained with STRING (11.0) at medium
22 confidence scores ≥ 0.4 .

1

2 **Figure 5. Validation of D0X475 and OmpH lysine-acetylated proteins in *V.***
3 ***alginolyticus* using Co-Immunoprecipitation and Western blotting.** D0X475 and
4 OmpH proteins were enriched by Co-IP with specific antibodies, followed by Western
5 blotting with D0X475 and OmpH proteins specific antibodies (above), and Western
6 blotting with anti-lysine acetylation antibodies (below).

7

8 **Figure 6. Comparison of *V. alginolyticus* acetylproteome with *V.***
9 ***parahaemolyticus*.** (A) Comparison of *V. alginolyticus* and *V. parahaemolyticus*
10 overlapped acetylated proteins, and KEGG pathway enrichment analysis with
11 overlapped proteins. (B) Comparison of *V. alginolyticus* and *V. parahaemolyticus*
12 overlapped acetylated peptides, and motif analysis with overlapped peptides.

13

14 **Figure 7.** Protein-protein interaction network of acetylated virulence factors in *V.*
15 *alginolyticus*. The size of the circle represents the number of modification sites.

16

17 **Supplemental Table S1. The identified acetylated proteins and sites in *V.***
18 ***alginolyticus*.**

19

20 **Supplemental Table S2. The identified peptides in *V. alginolyticus*.**

21

22 **Supplemental Table S3. List of *V. alginolyticus* virulence factors. The list was**

1 **generated based on VFDB database.**

1 Fig 1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

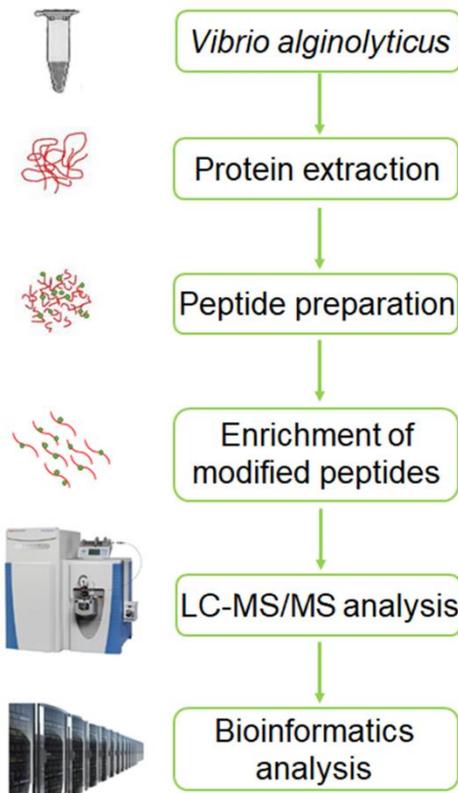
16

17

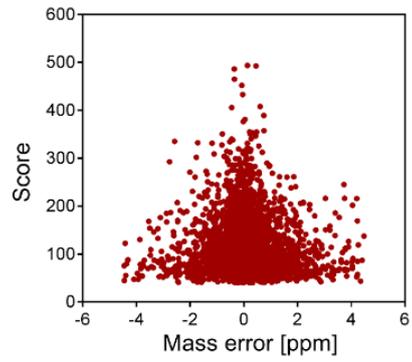
18

19

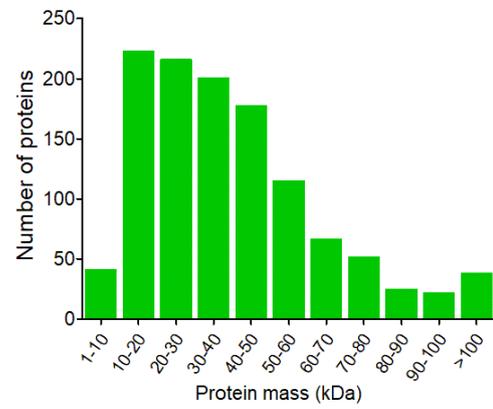
20



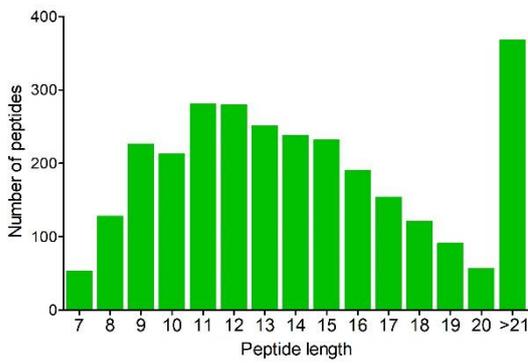
A



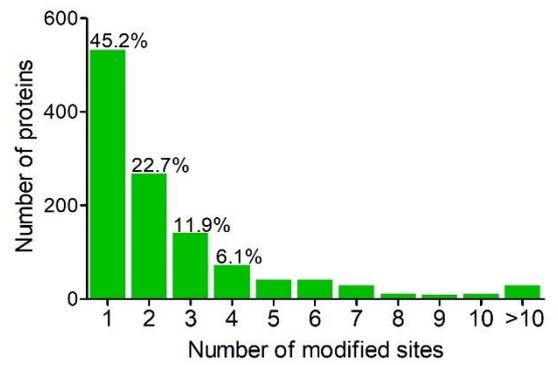
B



C

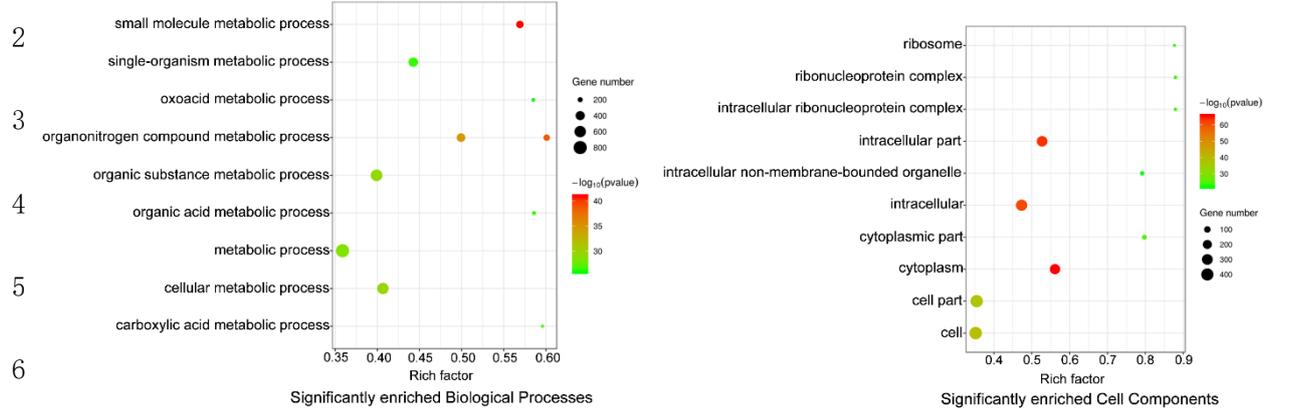


D



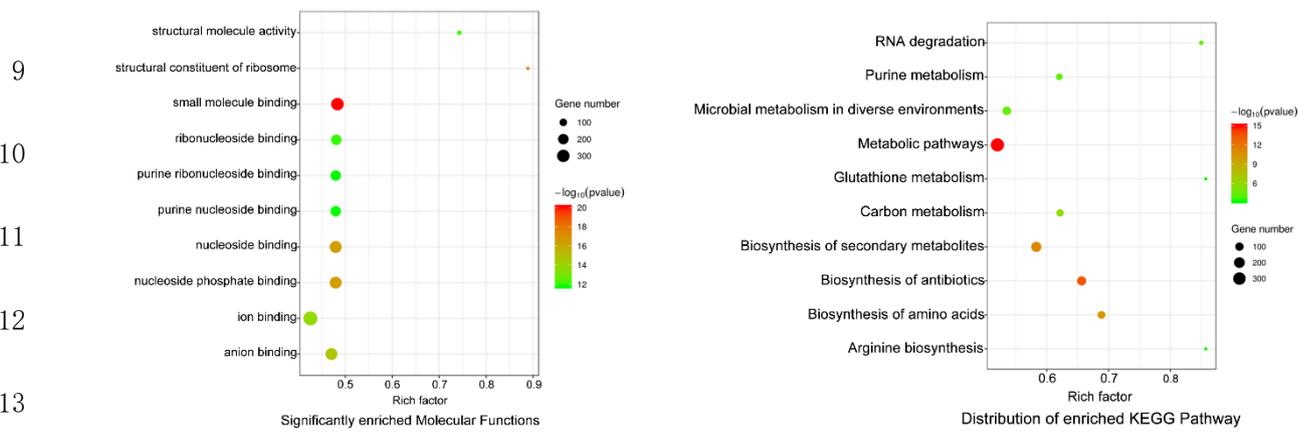
E

1 Fig 2



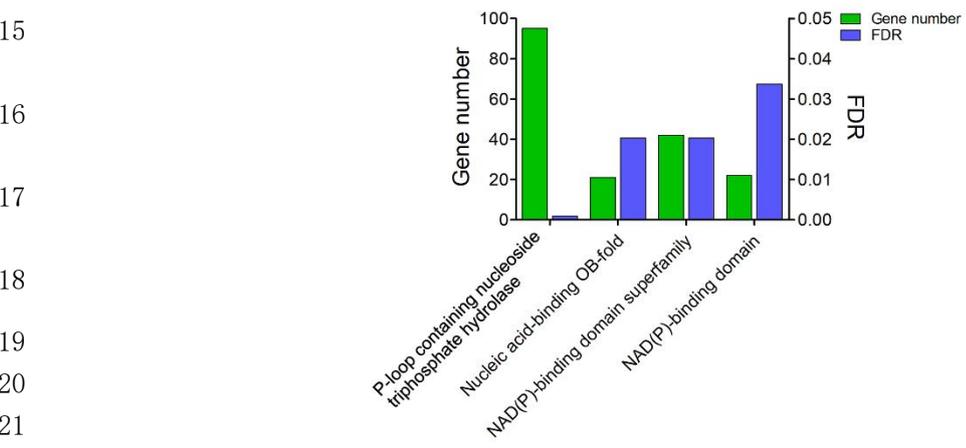
7 A

8 B



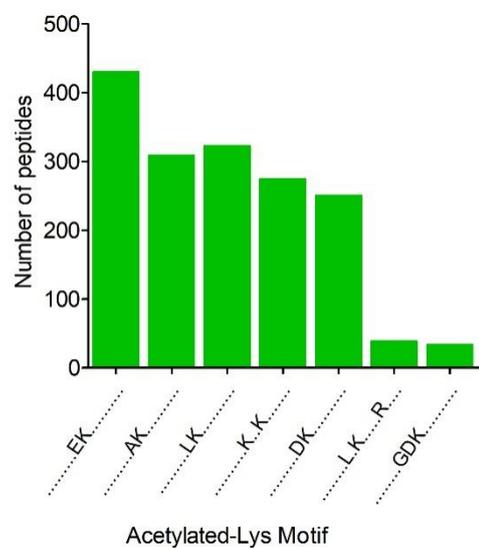
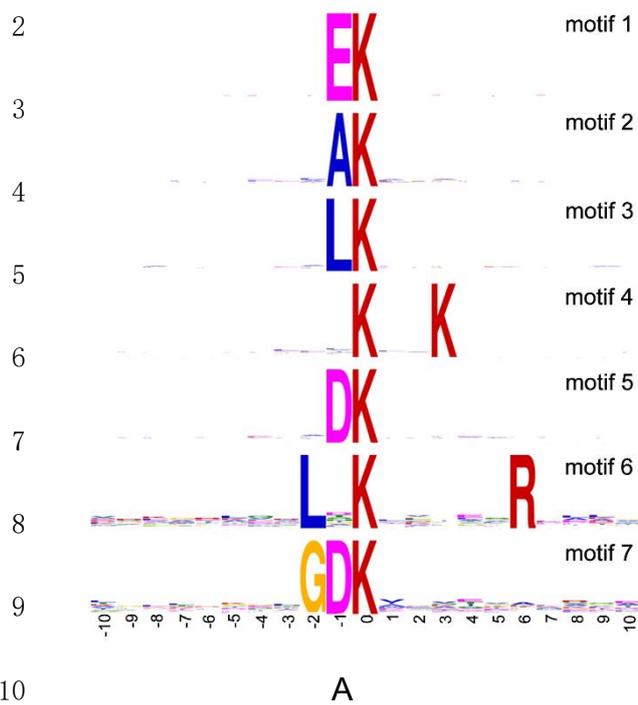
14 C

15 D



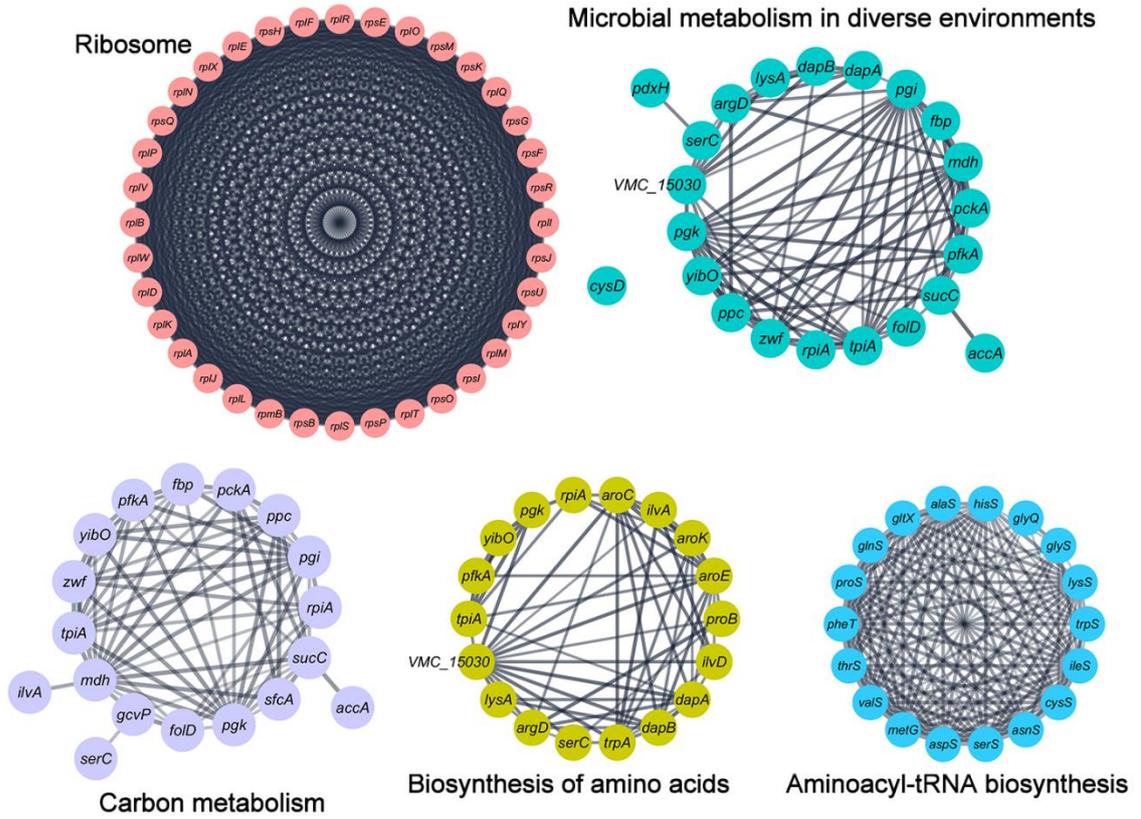
E

1 Fig 3



1 Fig 4

2



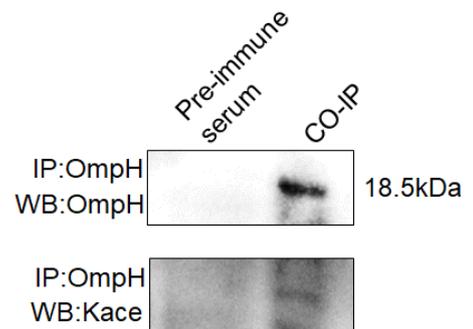
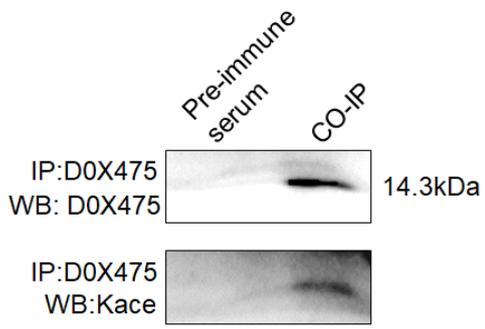
1 Fig 5

2

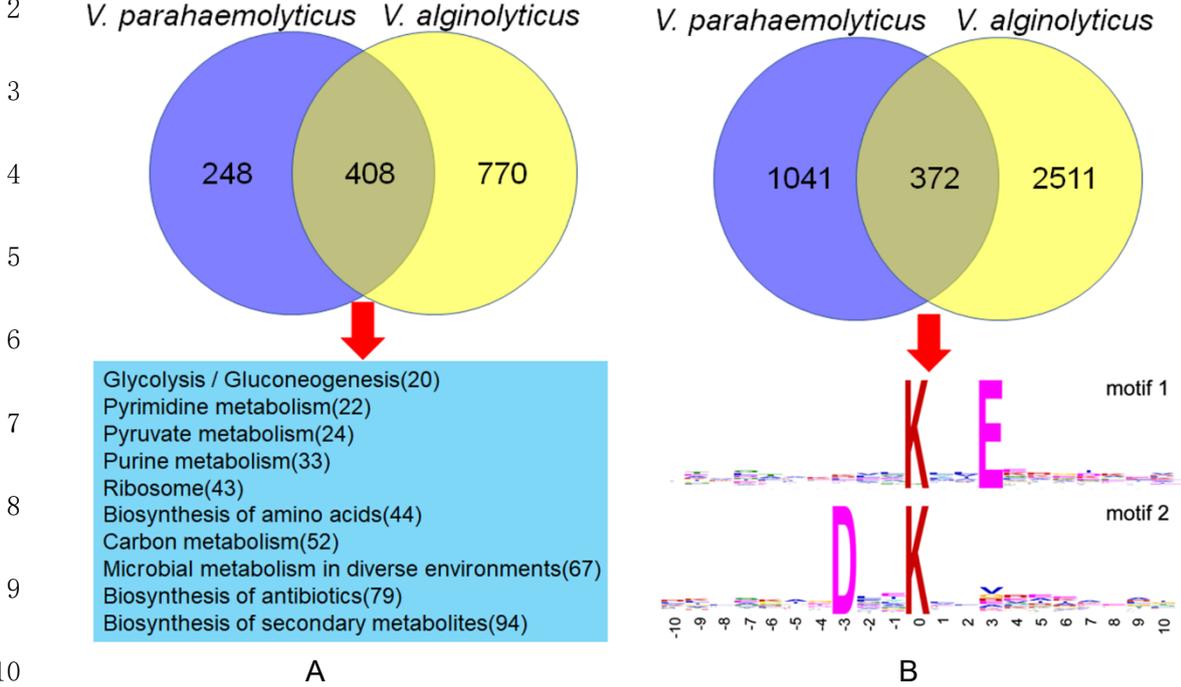
3

4

5



1 Fig 6



References

- [1] W. Soichi, K. Ryuichi, K. Masato, I. Noriyuki, M. Hiraki, Structural and functional biology for post translational modification and transport in eukaryotes, *Tanpakushitsu Kakusan Koso*. 53 (5) (2008) 628-631.
- [2] J.A. Cain, N. Solis, S.J. Cordwell, Beyond gene expression: The impact of protein post-translational modifications in bacteria, *J. Proteomics* 97 (2014) 265-286.
- [3] E.F. de Zoeten, L. Wang, K. Butler, U.H. Beier, T. Akimova, H. Sai, J.E. Bradner, R. Mazitschek, A.P. Kozikowski, P. Matthias, Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells, *Mol. Cell. Biol.* 31 (10) (2011) 2066-2078.
- [4] T.E. Tracy, L. Gan, Acetylated tau in Alzheimer's disease: An instigator of synaptic dysfunction underlying memory loss: Increased levels of acetylated tau blocks the postsynaptic signaling required for plasticity and promotes memory deficits associated with tauopathy, *Bioessays* 39 (4) (2017) 1600224.
- [5] J.Y. Pan, Z.C. Ye, Z.Y. Cheng, X.J. Peng, L.Y. Wen, F.K. Zhao, Systematic analysis of the lysine acetylome in *Vibrio parahaemolyticus*, *J. Proteome Res.* 13 (7) (2014) 3294-3302.
- [6] K. Zhang, S.Z. Zheng, J.S. Yang, Y. Chen, Z.Y. Cheng, Comprehensive profiling of protein lysine acetylation in *Escherichia coli*, *J. Proteome Res.* 12 (2) (2013) 844-851.
- [7] F.Y. Liu, M.K. Yang, X.D. Wang, S.S. Yang, J. Gu, J. Zhou, X.E. Zhang, J.Y. Deng, F. Ge, Acetylome analysis reveals diverse functions of lysine acetylation in *Mycobacterium tuberculosis*, *Mol. Cell. Biol.* 13 (12) (2014) 3352-3366.
- [8] J. Yan, R. Barak, O. A. Shainskaya, M. Eisenbach, In vivo acetylation of CheY, a response regulator in chemotaxis of *Escherichia coli*, *J. Mol. Biol.* 376 (5) (2008) 1260-1271.
- [9] J. Ren, Y. Sang, J. Lu, Y.F. Yao, Protein acetylation and its role in bacterial virulence, *Trends Microbiol.* 25 (9) (2017). 768-779.
- [10] L. Liu, G.Y. Wang, L.M. Song, B.N. Lv, W.X. Liang, Acetylome analysis reveals the involvement of lysine acetylation in biosynthesis of antibiotics in *Bacillus amyloliquefaciens*, *Sci. Rep.* 6 (2016) 20108.
- [11] K.C. Ravindra, B.R. Selvi, M. Arif, B.A. Reddy, G.R. Thanuja, S. Agrawal, S.K. Pradhan, N. Nagashayana, D. Dasgupta, T.K. Kundu, Inhibition of lysine acetyltransferase KAT3B/p300 activity by a naturally occurring hydroxynaphthoquinone, plumbagin, *J. Biol. Chem.* 284 (36) (2009) 24453-24464.
- [12] A. Bougdour, D. Maubon, P. Baldacci, P. Ortet, O. Bastien, A. Bouillon, J.C. Barale, H. Pelloux, R. Ménard, M.A. Hakimi, Drug inhibition of HDAC3 and epigenetic control of differentiation in *Apicomplexa parasites*, *J. Exp. Med.* 206 (4) (2009) 953-966.
- [13] L.N. Sun, Z.J. Yao, Z. Guo, L.S. Zhang, Y.Q. Wang, R.R. Mao, Y.X. Lin, Y.Y. Fu, X.M. Lin, Comprehensive analysis of the lysine acetylome in *Aeromonas hydrophila* reveals cross-talk between lysine acetylation and succinylation in LuxS, *Emerg. Microbes Infect.* (2019) accepted.
- [14] H.Y. Pang, L.M. Chen, R. Hoare, Y.C. Huang, Z.H. Wu, J.C. Jian, Identification of DLD, by immunoproteomic analysis and evaluation as a potential vaccine antigen against three *Vibrio* species in *Epinephelus coioides*, *Vaccine* 34 (9) (2016) 1225-1231.
- [15] S.H. Cai, Z.H. Wu, J.C. Jian, Y.S. Lu, Cloning and expression of the gene encoding an extracellular alkaline serine protease from *Vibrio alginolyticus* strain HY9901, the causative

-
- agent of vibriosis in *Lutjanus erythropterus* (Bloch), J. fish Dis. 30 (8) (2007) 493-500.
- [16] J. Wu, Y.H. Shi, X.H. Zhang, C.H. Li, M.Y. Li, J. Chen, Molecular characterization of an IL-1 β gene from the large yellow croaker (*Larimichthys crocea*) and its effect on fish defense against *Vibrio alginolyticus* infection, Zoological Res. 36 (3) (2015) 133-141.
- [17] H.Y. Pang, M.S. Qiu, J.M. Zhao, R. Hoare, S.J. Monaghan, D.W. Song, Y.S. Chang, J.C. Jian, Construction of a *Vibrio alginolyticus* *hopPmaJ* (*hop*) mutant and evaluation of its potential as a live attenuated vaccine in orange-spotted grouper (*Epinephelus coioides*), Fish Shellfish Immunol. 76 (2018) 93-100.
- [18] S.H. Cai, H.Y. Cheng, H.Y. Pang, J.C. Jian, Z.H. Wu, *AcfA* is an essential regulator for pathogenesis of fish pathogen *Vibrio alginolyticus*, Vet. Microbiol. 213 (2018) 35-41.
- [19] Z.J. Zhou, H.Y. Pang, Y. Ding, J. Cai, Y.C. Huang, J.C. Jian, Z.H. Wu, *VscO*, a putative T3SS chaperone escort of *Vibrio alginolyticus*, contributes to virulence in fish and is a target for vaccine development, Fish Shellfish Immunol. 35 (5) (2013) 1523-1531.
- [20] L.X. Huang, L.N. Guo, X.J. Xu, Y.X. Qin, L.M. Zhao, Y.Q. Su, Q.P. Yan, The role of *rpoS* in the regulation of *Vibrio alginolyticus* virulence and the response to diverse stresses, J. Fish Dis. 42(5) (2019) 703-712.
- [21] Y.Y. Chen, F.L. Wu, H. Pang, Y. J.F. Tang, S.H. Cai, J.C. Jian, Superoxide dismutase B (*sodB*), an important virulence factor of *Vibrio alginolyticus*, contributes to antioxidative stress and its potential application for live attenuated vaccine, Fish Shellfish Immunol. 89 (2019) 354-360.
- [22] Z.J. Yao, Z. Guo, Y.Q. Wang, W.X. Li, Y.Y. Fu, Y.X. Lin, W.X. Lin, X.M. Lin Integrated succinylome profiling and metabolomics reveal crucial role of S-ribosylhomocysteine lyase in quorum sensing and metabolism of *Aeromonas hydrophila*, Mol. Cell. Proteomics 18 (2) (2019) 200-215.
- [23] Z. Chen, L. Luo, R.F. Chen, H.H. Hu, Y.F. Pan, H.B. Jiang, X. Wan, H. Jin, Y.M. Gong, Acetylome profiling reveals extensive lysine acetylation of the fatty acid metabolism pathway in the diatom *Phaeodactylum tricornutum*, Mol. Cell. Proteomics 17 (3) (2018) 399-412.
- [24] Z.W. Pan, M.L. Wang, Z. Ye, S.J. Zhang, X.H. Xu, Global analysis of histone lysine acetylation and proteomic changes in EC109 cells treated with the histone deacetylase inhibitor FK228, Oncol. Lett. 15 (5) (2018) 7973-7980.
- [25] W.X. Li, Z.J. Yao, X.Y. Zhang, F. Huang, W.X. Lin, X.M. Lin, Global protein expression profile response of planktonic *Aeromonas hydrophila* exposed to chlortetracycline, World J. Microbiol. Biotechnol. 33 (4) (2017) 68.
- [26] M. Kohl, S. Wiese, B. Warscheid, Cytoscape: software for visualization and analysis of biological networks, Methods Mol. Biol. 696 (2011) 291-303.
- [27] B. Liu, D.D. Zheng, Q. Jin, L.H. Chen, J. Yang, VFDB 2019: a comparative pathogenomic platform with an interactive web interface, Nucleic acids Res. 47(D1) (2018) D687-D692.
- [28] Z.X. Cheng, C. Guo, Z.G. Chen, T.C. Yang, J.Y. Zhang, J. Wang, J.X. Zhu, D. Li, T.T. Zhang, H. Li, Glycine, serine and threonine metabolism confounds efficacy of complement-mediated killing, Nat. Commun. 10 (1) (2019) 3325.
- [29] Y.Q. Wang, X.Y. Wang, F.M. Ali, Z.Q. Li, Y.Y. Fu, X.J. Yang, W.X. Lin, X.M. Lin, Comparative extracellular proteomics of *Aeromonas hydrophila* reveals iron-regulated secreted proteins as potential vaccine candidates, Front. Immunol. 10 (2019) 256.
- [30] M. Hui, J. Cheng, Z.L. Sha, First comprehensive analysis of lysine acetylation in

Alvinocaris longirostris from the deep-sea hydrothermal vents, BMC Genomics 19(1) (2018) 352.

[31] S.Y. Zhou, Q.Q. Yang, C.F. Yin, L. Liu, W.X. Liang, Systematic analysis of the lysine acetylome in *Fusarium graminearum*, BMC genomics 17(1) (2016) 1019.

[32] Y.B. Su, B. Peng, H. Li, Z.X. Cheng, T.T. Zhang, J.X. Zhu, D. Li, M.Y. Li, J.Z. Ye, C.C. Du, Pyruvate cycle increases aminoglycoside efficacy and provides respiratory energy in bacteria, Proc. Nati. Acad. Sci. U S A 115 (7) (2018) E1578-E1587.

[33] J.M. Peters, A. Colavin, H. Shi, T.L. Czarny, M.H. Larson, S. Wong, J.S. Hawkins, C.H. Lu, B.-M. Koo, E. Marta, A comprehensive, CRISPR-based functional analysis of essential genes in bacteria, Cell 165 (6) (2016) 1493-1506.

[34] L.X. Xie, X.B. Wang, J. Zeng, M.L. Zhou, X.K. Duan, Q.M. Li, Z. Zhang, H.P. Luo, L. Pang, W. Li, G.J. Liao, X. Yu, Y.X. Li, H.R. Huang, J.P. Xie, Proteome-wide lysine acetylation profiling of the human pathogen *Mycobacterium tuberculosis*, Int. J. Biochem. Cell Biol. 59 (2015) 193-202.

[35] D.D. Leipe, Y.I. Wolf, E.V. Koonin, L. Aravind, Classification and evolution of P-loop GTPases and related ATPases, J. Mol. Biol. 317 (1) (2002) 41-72.

[36] E.A. Sickmier, D. Brekasis, S. Paranawithana, J.B. Bonanno, M.S. Paget, S.K. Burley, C.L. Kielkopf, X-ray structure of a Rex-family repressor/NADH complex insights into the mechanism of redox sensing, Structure 13 (1) (2005) 43-54.

[37] M.F. Chou, D. Schwartz, Biological sequence motif discovery using motif - x, Curr. Protoc. Bioinformatics 35 (1) (2011) 15-24.

[38] O. Hiroki, K. Kwang, M. Ryoji, K. Seiki, Acetylome with structural mapping reveals the significance of lysine acetylation in *Thermus thermophilus*, J. Proteome Res. 12 (9) (2013) 3952-3968.

[39] R. Qin, Y. Sang, J. Ren, Q.F. Zhang, S.X. Li, Z.L. Cui, Y. F. Yao, The bacterial two-hybrid system uncovers the involvement of acetylation in regulating of Lrp activity in *Salmonella typhimurium*, Front. Microbiol. 7 (2016) 1864.

[40] T. Ouidir, P. Cosette, T. Jouenne, J. Hardouin, Proteomic profiling of lysine acetylation in *Pseudomonas aeruginosa* reveals the diversity of acetylated proteins, Proteomics 15 (13) (2015) 2152-2157.

[41] T. Kentache, T. Jouenne, E. Dé, J. Hardouin, Proteomic characterization of N α - and N ϵ -acetylation in *Acinetobacter baumannii*, J. Proteomics 144 (2016) 148-158.

[42] S.P. Bernier, D.T. Nguyen, P.A. Sokol, A LysR-type transcriptional regulator in *Burkholderia cenocepacia* influences colony morphology and virulence, Infect. Immun. 76 (1) (2008) 38-47.

[43] R.Q. Tang, G. Luo, L.M. Zhao, L.X. Huang, Y.X. Qin, X.J. Xu, et al. The effect of a LysR-type transcriptional regulator gene of *Pseudomonas plecoglossicida* on the immune responses of *Epinephelus coioides*, Fish shellfish Immunol. 2019 89:420-7.

[44] A.K. Heroven, P. Dersch, RovM, a novel LysR-type regulator of the virulence activator gene *rovA*, controls cell invasion, virulence and motility of *Yersinia pseudotuberculosis*, Mol. Microbiol. 62 (5) (2010) 1469-1483.

[45] V.B. Srinivasan, A. Mondal, M. Venkataramaiah, N.K. Chauhan, G. Rajamohan, Role of oxyR^{KP}, a novel LysR-family transcriptional regulator, in antimicrobial resistance and virulence in *Klebsiella pneumoniae*, Microbiology 159 (7) (2013) 1301-1314.

-
- [46] K.H. Turner, I. Vallet-Gely, S.L. Dove, Epigenetic control of virulence gene expression in *Pseudomonas aeruginosa* by a LysR-type transcription regulator, *PLoS Genet.* 5 (12) (2009) e1000779.
- [47] F. Yang, S. Qian, F. Tian, H. Chen, W. Huthins, C.H. Yang, C. He, The GGDEF-domain protein GdpX1 attenuates motility, exopolysaccharide production, and virulence in *Xanthomonas oryzae pv. oryzae*, *J. Appl. Microbiol.* 120 (6) (2016) 1646-1657.
- [48] C. Wei, W.D. Jiang, M.R. Zhao, J.J. Ling, X. Zeng, J. Deng, D.L. Jin, J.M. Dow, W.X. Sun, A systematic analysis of the role of GGDEF-EAL domain proteins in virulence and motility in *Xanthomonas oryzae pv. oryzicola*, *Sci. Rep.* 6 (1) (2016) 23769.