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# AcfA is an essential regulator for pathogenesis of fish pathogen *Vibrio alginolyticus*

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ABSTRACT

*V. alginolyticus* is an important opportunistic pathogen which causes vibriosis in aquatic animals. AcfA, as an accessory colonization factor, is hypothesized to be involved in the pathogenesis of infection. In this study, a mutant strain with an in-frame deletion removed nucleotides 86 to 561 of the acfA gene was constructed to reveal the role of AcfA in the physiology and virulence from *V. alginolyticus*. An acfA mutant showed a similar growth level, an obvious decrease in swarming motility and the activity of ECPase, a higher $LD_{50}$ value by intraperitoneal injection of grouper fish compared to that of the wild-type. Furthermore, the deletion of acfA could enhance the level of biofilm formation and suppress the polar flagellum forming. The comparative proteomic analysis demonstrated the deletion mutation of acfA could up-regulate the expression of 4 proteins of p4alcd, deoD, phb and DctP, and down-regulate the expression of 8 proteins of Clp, hpV36980, ABCtp, pepD, arA, aggp, fla and ompA compared to that of the wild-type. The analysis of RT-qPCR showed the mRNA levels of DctP and deoD were significantly induced, and the mRNA levels of pepD, arA, fla and ompA were significantly reduced in acfA mutant compared with the wild-type. The results suggest that acfA may contribute to the overall success in the pathogenesis of *V. alginolyticus* by regulating the expression of some relevant genes.

## 1. Introduction

The Gram-negative bacterium *Vibrio alginolyticus* is natural lives in marine environments and a part of normal marine flora. However, it has been considered to be one of the important epizootic pathogens to several marine animals. In the coastal provinces of south China, *V. alginolyticus* has been reported to be the dominant causative media of high-mortality outbreaks of vibriosis in several fish and shellfish species (Cai et al., 2013). Despite the boom of fish farming in south China, the whole industry was badly hampered by the fish mortality because of vibrosis caused by *V. alginolyticus*. It is also a pathogenic bacterium associated with ear infections and gastrointestinal diseases in humans (Austin, 2010; Uh et al., 2001). Nevertheless, its exact role as an enteric pathogen is unclear. It is well known that the use of antimicrobial substances has some drawbacks in the prevention of vibrosis, such as emergence of resistant strains of pathogens. In addition, clinical isolates usually have high lethality in fish and high cytotoxicity to various cultured cells and experimental animals compared with environmental isolates (Cao et al., 2010; Lu et al., 2011). Pathogenicity of *V. alginolyticus* is associated with possession of some virulence factors and the ability to co-ordinately regulate the expression of these factors in response to environmental stimuli, such as alkaline serine protease, VscO, ToxR, having been reported in vivo and in vitro (Cai et al., 2007; Chen et al., 2012; Zhou et al., 2013).

The accessory colonization factors (Acfs) including AcfA, AcfB, AcfC and AcfD locate on a 29-kb Xba I chromosomal fragment in V. cholerae, and are the essential intestinal colonization factors by Vibrio in the infant mouse model system, are physically linked to toxin co-regulated pilus and is under the regulatory cascade that directs the synthesis of cholera toxin and other proteins required for colonization in V. cholera (Withey and Dirita, 2005; Sharma et al., 2008). The acfA gene is one of four closely linked genes encodes components of the accessory colonization factors, and its product is required for efficient colonization of V. cholerae in the intestine as its disruption leads to reduce colonization (Peterson and Mekalanos, 1988). Currently, Acfs of V. cholerae and their regulation mechanism in intestine colonization have been studied and the detailed information on structural and functional characteristics are well known (Everiss et al., 1994; Hughes et al., 1995), but there are little reports that Acfs have been studied in *V. alginolyticus*.

Proteomic technique is a useful method in studying proteins differentially expressed under altered conditions (Sprenger et al., 2004). Furthermore, the proteome of related *V. alginolyticus* has been analyzed,

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and several proteins possibly linked to pathogenesis have been characterized (Xu et al., 2005; Pang et al., 2013). In the current study, a mutant strain of *V. alginolyticus* with an in-frame deletion of the acfA gene was constructed, and then physiology and virulence of mutant strain was observed. Furthermore, employing a similar proteomic technique, we compared the proteomes of the wild-type and acfA mutant to identify proteins possibly regulated by acfA gene, and to determine if some of those proteins are also involved in virulence. Finally, the Real-time quantitative PCR validated the mRNA expression levels of those proteins identified in the proteomes.

## 2. Materials and methods

### 2.1. Ethics statement

The grouper fish (*Epinephelus coioides*) used for virulence tests in this study were cultured animals. All the experiments were carried out in strict accordance with the regulations of local government and approved by the Ethics Committee of Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals.

### 2.2. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are shown in Table 1. *V. alginolyticus* strain HY9901 was isolated from the diseased grouper fish in Hainan, China, and maintained in our laboratory. *V. alginolyticus* was grown in tryptic soy broth (TSB, Huankai, Guangzhou, China) supplemented with 2% NaCl at 25 °C, while *Escherichia coli* strains were cultured in Luria broth (LB, Huankai, Guangzhou, China) at 37 °C. When required, the appropriate antibiotics were added: ampicillin (Amp, 100 μg $ml^{-1}$) or chloramphenicol (Cm, 30 μg $ml^{-1}$).

### 2.3. Construction of the acfA mutant

Based on the sequence of acfA gene of *V. alginolyticus* strain HY9901 cloned by our laboratory, the primers for construction of the acfA mutant were designed (Table 2). The PCR-amplified DNA fragment used for constructing the in-frame deletion mutation of acfA was generated by overlap PCR (Ho et al., 1989; Milton et al., 1996). Briefly, two PCR fragments were obtained from *V. alginolyticus* strain HY9901 genomic DNA with the primer pairs of AcfA-for/AcfA-int-rev and AcfA −int-for/AcfA-rev by using the pfu Taq polymerase (Promega, Madison, WI). The resulting products generated a 141 bp fragment containing the DNA upstream of acfA and a 122 bp fragment containing the DNA downstream of the acfA. A 15-bp overlap in the sequences (Italic) permitted amplification of a 253 bp product containing a deletion from nucleotides 86 to 561 of acfA during a second PCR with primers of AcfA-for and AcfA-rev, which were introduced at a Kpn I or Sac I restriction site (underlined), respectively. The resulting product was digested with Kpn I or Sac I, then inserted into the same sites of the suicide plasmid pRE112 which carried a sacB sucrose-sensitivity gene and conferred chloramphenicol resistance, generating the recombinant plasmid pRE112-acfA transformed into E. coli MC1061 λpir and subsequently S17-1 λpir. The recombinant plasmid pRE112-acfA was sequenced to confirm the correct construct. E. coli S17-1 λpir containing the plasmid pRE112-acfA was conjugated with wild-type *V. alginolyticus* strain HY9901. Recipient cells were plated on TSA supplemented with 1% glucose and the antibiotics chloramphenicol to select the clone pRE112-acfA that had integrated the vector by a single crossover of allelic exchange. Antibiotic-resistant colonies were isolated, grown in

**Table 2**
Sequences of primers used in this study.

| Name | Sequence (5′ − 3′) | Targeting sequence | Product size (bp) |
|---|---|---|---|
| AcfA-for | CGGGGTACCTTCTCACAAAATGCGTAT (KpnI) | Upstream of acfA | 141 |
| AcfA-int-rev | GGAGCCTAGTCCATATTCCAG | | |
| AcfA −i-nt-for | TATGGACTAGGCTCCATAGGTGTAAAC | Downstream of acfA | 122 |
| AcfA-rev | CGGAGCTCGTTAACAATACTCTTTTCAT (SacI) | | |
| AcfA-F1 | CGCGGATCCGCACATAAGTACATTTCT (BamHI) | Flanking region and ORF sequence of acfA | 707 |
| AcfA-F2 | ACATGCATGCTACTCTTTTCATTATCCT (SphI) | | |
| DctP-F | GAAAAGCGCGTCAACGAAGA | qRT-PCR | 239 |
| DctP-R | TTTTCACCCGCTTCCGAGTT | | |
| pepD-F | AGGCATCTACGGTCACAAGC | qRT-PCR | 195 |
| pepD-R | GCTTCAAGAAGCGCAACCAT | | |
| arA-F | CTGCGGATGGCAAAATCGAG | qRT-PCR | 157 |
| arA-R | ACGTGCATACGGGTTGAGTT | | |
| fla-F | TGGTAAGAGCTACGCAGCAG | qRT-PCR | 201 |
| fla-R | TCTTCGCCAACAGAGGCTTT | | |
| deoD-F | GTTCGCAACATGTTCGGCTT | qRT-PCR | 162 |
| deoD-R | ACGTACTGCACCACAGCTAC | | |
| ompA-F | CGGAAGAAGTGATCGAGCCA | qRT-PCR | 249 |
| ompA-R | CAACAGATTGAGCACGACGC | | |

**Table 1**
Plasmids and bacterial strains used in this study.

| Strains and plasmids | Phenotype | Source or references |
|---|---|---|
| E. coli strains | | |
| DH5α | SupE44ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1 | Sangon |
| MC1061 λpir | lacY1 galK2 ara-14 xyl-5 supE44 λpir | Rubirés et al. (1997) |
| MC1061-pRE112-acfA | MC1061 containing plasmid of pRE112- acfA, Cmr | This study |
| S17-1 λpir | $Tp^r$ $Sm^r$ recA thi pro $hsdR^-M^+$RP4:2-Tc:Mu:Km Tn7 λpir | Simon et al. (1983) |
| S17-1-pRE112-acfA | S17-1 containing plasmid of pRE112- acfA, Cmr | This study |
| HY9901 | Wild-type, isolated from the diseased fish | Cai et al. (2007) |
| HY9901-pRE112-acfA | HY9901 containing plasmid of pRE112-acfA, $Cm^r$ | This study |
| HY9901ΔacfA | HY9901 carrying an in-frame deletion of $acfA_{86–561}$ | This study |
| C−HY9901ΔacfA | HY9901ΔacfA containing plasmid of pACYC184-acfA, $Cm^r$ | This study |
| Plasmid | | |
| pMD18-T | Cloning vector; $Amp^r$ | TaKaRa |
| pRE112 | pGP704 suicide plasmid, pir dependent, oriT, oriV, sacB, $Cm^r$ | Edwards et al. (1998) |
| pRE112-acfA | pRE112 containing acfA gene in-frame deletion of $acfA_{86–561}$, $Cm^r$ | This study |
| pACYC184 | $Cm^r$, $Tc^r$ | Amersham |
| pACYC184-acfA | pACYC184 containing acfA gene, $Cm^r$ | This study |