The Involvement of Thiamine Uptake in the Virulence of Edwardsiella piscicida

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Abstract: Edwardsiella piscicida is a pathogenic bacterium, which can infect a number of fish species and cause a disease termed edwardsiellosis, threatening global fish farming with high prevalence and mortality. Thiamine (Vitamin B1), functioning in the form of thiamine pyrophosphate (TPP), is essential for almost all organisms. Bacteria acquire TPP by biosynthesis or by transportation of exogenous thiamine. TPP availability has been associated with bacterial pathogenicity, but the underlying mechanisms remain to be discovered. The role of thiamine in the pathogenicity of E. piscicida is unknown. In this study, we characterized a thiamine transporter (TT) operon in E. piscicida. The deletion of the TT operon resulted in an intracellular TPP lacking situation, which led to attenuated overall pathogenicity, impaired abilities associated with motility and host cell adhesion, as well as decreased expression of certain flagellar and adhesion genes. Moreover, TPP starvation led to intracellular c-di-GMP reduction, and introducing into the TPP-suppressed mutant strain an exogenous diguanylate cyclase for c-di-GMP synthesis restored the virulence loss. Taken together, this work reveals the involvement of thiamine uptake in the virulence regulation of E. piscicida, with c-di-GMP implicated in the process. These finding could be employed to explore potential drug targets against E. piscicida.

Keywords: Edwardsiella piscicida; thiamine; transporter; c-di-GMP; virulence

1. Introduction

Edwardsiella piscicida is a Gram-negative, facultatively anaerobic bacterium. The genus Edwardsiella is currently composed of five species, including E. piscicida, E. ictaluri, E. anguillarum, E. tarda, and E. hoshiniae [1]. E. piscicida is a relatively new species that contains the pathogenic fish isolates formerly considered to be E. tarda [2]. E. piscicida was isolated from freshwater, estuary, marine environments, and animal tissues. It can infect a wide range of animals, including fish, reptiles, birds, and mammals [1,5]. It is a major threat to global fish farming by causing edwardsiellosis, a severe disease featured by ascites, hernia, exophthalmia, and severe lesions of internal organs in numerous fish species, such as channel catfish (Ictalurus punctatus), common carp (Cyprinus carpio), Japanese eel (Anguilla japonica), Japanese flounder (Paralichthys olivaceus), largemouth bass (Micropterus salmoides), striped bass (Morone saxatilis), and turbot (Scophthalmus maximus) [3,4].

The known virulence factors in E. piscicida include flagella, adhesion factors such as adhesins and invasins, serum resistance proteins, and the type III and type VI secretion systems [5–13]. During infection, E. piscicida is motivated by the rotation of flagella in response to host cues and attaches to the entry sites on the host, such as the skin, gastrointestinal, and gills, via adhesion factors [14,15]. The pathogen then penetrates the tissue barrier,
replicates inside host cells including phagocytes, and sabotages host immune responses with an armory of virulence factors and their mediators [4,14,16,17].

Thiamine is an essential microelement required for almost all organisms [18,19]. Its active form, thiamine pyrophosphate (TPP), participates in various biological processes by acting as a coenzyme or through non-coenzyme mechanisms [18,20]. Bacteria acquire thiamine and TPP by de novo synthesis and/or by uptake via transporters. Bacterial transporters of thiamine can be classified as ABC-type transporters, energy coupling factor (ECF) importers, NiaP transporters, and PnuT transporters [21]. The Salmonella thiBPQ operon encodes a representative ABC-type thiamine uptake system, which comprises a periplasmic thiamine binding protein, a transmembrane channel protein, and an ATPase, together forming an ATP-binding cassette-type transporter [22–24]. Two reports indicated an association of thiamine transport with bacterial pathogenicity. In these reports, the mutation of a thiamine transporter in Listeria monocytogenes and Verticillium dahliae resulted in reduced bacterial virulence [25,26]. Otherwise, the role of thiamine uptake in the pathogenicity of bacteria, including E. piscicida, remains to be investigated.

In carbohydrate metabolism, TPP functions as a cofactor for enzymes such as transketolase, α-ketoacid decarboxylase, α-ketoacid dehydrogenase, and acetolactate synthase [20]. Transketolase is a key enzyme in the pentose phosphate pathway, which in bacteria provides the pentose skeleton for the biosynthesis of cyclic-di-GMP (c-di-GMP) [27]. C-di-GMP is a second messenger that mediates many aspects of bacterial lifecycle, such as motility, biofilm formation, replication, and virulence [28,29]. Bacterial c-di-GMP is synthesized by diguanylate cyclases (DGCs) and degraded by c-di-GMP-specific phosphodiesterases (PDEs) [30]. The concentration dynamic of c-di-GMP is controlled by an array of DGCs and PDEs upon intracellular and/or environmental cues, and subsequently converted to physiological responses via various c-di-GMP binding effectors, including RNA and proteins [29]. The RNA effectors refer to Class I and Class II c-di-GMP riboswitches, which control the downstream gene transcription and translation, respectively [31]. The known protein receptors for c-di-GMP include transcriptional regulators, PilZ proteins, YajQ proteins, and ATPases [29]. These effectors connect c-di-GMP signaling to gene expression and protein functionality, and interplay with other molecular pathways such as quorum sensing [32]. In pathogenic bacteria, a number of virulence genes and traits are regulated by c-di-GMP signaling in a dynamic and varied fashion. In general, low levels of c-di-GMP seem to be programmed for acute infection and associated with the bacterial ability to destroy or penetrate host cells [29]. In contrast, high levels of c-di-GMP seem to favor bacterial colonization of host tissues [29]. The genome of E. piscicida contains at least 11 c-di-GMP associated genes, encoding DGCs, PDEs, and an effector protein [33]. However, neither c-di-GMP signaling nor its possible role in the pathogenicity of E. piscicida has been reported thus far.

In this work, we characterized a thiBPQ-type thiamine transporter operon in E. piscicida and investigated its contribution to pathogenicity. We then identified c-di-GMP as a mediating molecule underlying the virulence regulation of thiamine uptake. Our results suggest that thiamine acquisition could be targeted for the development of new therapies against E. piscicida.

2. Results
2.1. E. piscicida Thiamine Transporter (TT) Operon Is Required for In Vivo Dissemination of E. piscicida

E. piscicida ZW1 possesses a thiBPQ thiamine transporter operon, which is 100% identical to that of E. piscicida EIB202. The sequences of thiB (ETAE_0610), thiP (ETAE_0609), and thiQ (ETAE_0608) overlap and form the coding part of the operon. The deduced amino acid sequences of ThiB, ThiP, and ThiQ are 331, 535, and 237 residues in length, and possess the feature domains of bacterial extracellular solute-binding protein (Pfam ID: PF01547), binding-protein-dependent transport system inner membrane component (Pfam ID: PF00528), and ATP-binding cassette transporter (Pfam ID: PF00005), respectively.
ThiB, ThiP, and ThiQ share 72.51% to 68.88%, 76.82% to 71.06%, and 68.49% to 60.85% sequence identities, respectively, with their homologues in *Salmonella enterica*, *Escherichia coli*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Serratia liquefaciens*, and *Yersinia kristensenii* (Supplementary Figure S1). The *thiBPQ* operon was named TT for short.

To inspect the functional importance of the TT operon, an isogenic mutant of ZW1, ZW1ΔTT, was constructed, in which almost the entire TT operon, from the 28th amino acid residue of ThiB to the 205th residue of ThiQ, was deleted markerlessly. The thiamine level in ZW1ΔTT was analyzed using a previously verified thiamine sensing reporter plasmid [34]. Compared to ZW1, ZW1ΔTT produced significantly enhanced GFP fluorescence signal, implying a relative lack of TPP in ZW1ΔTT (Figure 1A). This result indicates that the TT operon is essential to thiamine transport. Growth analysis showed that ZW1ΔTT exhibited a growth curve similar to that of ZW1 (Figure 1B). In vivo infection showed that following inoculation into turbot, the numbers of ZW1ΔTT in the spleen and kidney of the fish were similar to that of ZW1 at 12 and 24 hpi, but were significantly lower than that of ZW1, as well as the TT complement strain ZW1ΔTTC at 48 hpi (Figure 1C,D). In addition, the mortality-inducing capacities of ZW1ΔTT and ZW1 were also compared. The results showed that at the same dose of infection, both ZW1ΔTT and ZW1 caused 100% mortality; however, ZW1ΔTT-induced death was much delayed than that induced by ZW1 (Supplementary Figure S2). These results indicate that thiamine uptake is required for the pathogenicity of *E. piscicida*.

Figure 1. The effects of *thiBPQ* knockout on the TPP content, growth, and infection of ZW1. (A) The intracellular TPP levels of ZW1 and ZW1ΔTT were analyzed with a reporter plasmid. The higher the fluorescence intensity, the lower the TPP content. (B) Growth curves of ZW1 and ZW1ΔTT in Luria–Bertani broth (LB) medium. (C, D) Turbot were infected with ZW1, ZW1ΔTT, or ZW1ΔTTC, and bacterial recoveries from spleen (C) and kidney (D) were determined by plate count at different time points. Data are presented as means ± SD, n = 3. **p < 0.01. Data marked with different letters have statistically significant differences (p < 0.05).
2.2. TT Knockout Reduces E. piscicida Motility and Flagellar Gene Expression

Soft agar swimming assay showed that ZW1ΔTT displayed apparently reduced motility in comparison to ZW1 or ZW1ΔTTc (Figure 2A, Supplementary Figure S3). Since flagella play an important role in motility, the expressions of flhB, flhD, flgA, flgB, fliC1, fliD, and fliF, the first genes of 7 flagellar operons, were analyzed by qRT-PCR. The results showed that the expressions of fliF, flhB, and fliC1 in ZW1ΔTT were significantly repressed compared to those in ZW1 or ZW1ΔTTc (Figure 2B–D, Supplementary Figure S4). Moreover, the expressions of the other genes in the fliF operon (fliG, fliH, fliI, fliJ, fliK, fliL, fliM, fliN, and fliO), the flhB operon (flhA and flhE), and the fliC operon (fliC2, fliA, and fliZ) were also significantly reduced in ZW1ΔTT (Figure 2B–D). These results demonstrate a positive effect of thiamine uptake on the motility of E. piscicida, which may be at least partially through the transcriptional regulation of the flagellar genes.

Figure 2. The effects of TT knockout on bacterial motility and flagellar gene expression. (A) Swimming abilities of ZW1, ZW1ΔTT, and ZW1ΔTTc were compared using LB medium containing 0.3% (w/v) agar. (B–D) The expression of flagellar genes of the fliF (B), flhB (C), and fliC (D) operons in ZW1, ZW1ΔTT, and ZW1ΔTTc were measured by qRT-PCR with topA as an internal reference. For the convenience of comparison, the expression levels in ZW1 were normalized as 1. Data are presented as means ± SD, n = 3. Data marked with different letters have statistically significant differences (p < 0.05).
2.3. TT Knockout Impairs *E. piscicida* Ability to Attach Host Cells

Cellular infection study showed that when turbot peripheral blood leukocytes (PBLs) were infected with ZW1, ZW1ΔTT, or ZW1ΔTTc for 2 h at an MOI of 1:1, the majority of the bacteria were still at the stage of cellular attachment (Supplementary Figure S5). Compared to ZW1, ZW1ΔTT exhibited a significantly decreased ability to attach to PBLs (Figure 3A). qRT-PCR analysis of the expression of eight adhesion factor genes showed that the mRNA levels of two genes, i.e., ETAE_1902 and ETAE_2842, were significantly lower in ZW1ΔTT than in ZW1 or ZW1ΔTTc (Figure 3B,C, Supplementary Figure S6). Furthermore, overexpression of ETAE_1902 in ZW1ΔTT significantly increased bacterial adhesion to PBLs to the extent comparable to that of ZW1 (Figure 3D). These results indicate that thiamine uptake participates in bacterial attachment to host cells in a manner that involves the transcriptional regulation of the adhesin gene ETAE_1902.

**Figure 3.** The effects of TT knockout on bacterial adhesion to fish cells and expression of adhesin and invasin genes. (A) Turbot peripheral blood leukocytes (PBLs) were incubated with ZW1, ZW1ΔTT, or ZW1ΔTTc for 2 h, and the number of the cell-attached bacteria was determined. (B,C) The expression of the adhesin/invasin genes ETAT_1902 (B) and ETAE_2842 (C) in ZW1, ZW1ΔTT, and ZW1ΔTTc were measured by qRT-PCR with topA as an internal reference. For the convenience of comparison, the expression levels in ZW1 were normalized as 1. (D) Turbot PBLs were incubated with ZW1, ZW1ΔTT, or ZW1ΔTT overexpressing ETAE_1902 or ETAT_2842 for 2 h, and the number of the cell-attached bacteria was determined. Data are presented as means ± SD, n = 3. Data marked with different letters have statistically significant differences (p < 0.05).
2.4. C-di-GMP Is Involved in the Thiamine Uptake-Associated Virulence Regulation of *E. piscicida*

The effect of TT knockout on the intracellular level of c-di-GMP was examined with a c-di-GMP sensing reporter plasmid [35]. Compared to ZW1, ZW1TT displayed a significantly more intense fluorescence signal, indicating a lower amount of c-di-GMP in ZW1TT (Figure 4). To assess the importance of c-di-GMP to the virulence associated traits of ZW1, the strain ZW1TT-adrA was constructed, which is ZW1TT overexpressing *adrA*, a DGC gene with certified function in c-di-GMP synthesis [36]. qRT-PCR showed that compared to ZW1TT, the expressions of the *fliF*, *flhB*, and *fliC* operons and the adhesin gene ETAE_1902 were all significantly increased in ZW1TT-adrA to the levels comparable to those in ZW1 (Figure 5A–C and Figure 6A). Consequently, the motility and host cell adhesion of ZW1TT-adrA were also increased (Figure 5D, Supplementary Figure S7, Figure 6B). Together these results suggest that thiamine uptake likely contributes to the pathogenicity of *E. piscicida* through c-di-GMP.

**Figure 4.** The effect of TT knockout on c-di-GMP level. The intracellular c-di-GMP content in ZW1 and ZW1TT was analyzed with a reporter plasmid. The lower the fluorescence intensity, the lower the c-di-GMP content. Data are presented as means ± SD, *n* = 3. **p < 0.01.

**Figure 5.** Cont.
Figure 5. Thiamine uptake affects flagellar gene expression and bacterial motility through c-di-GMP. The gene expression of the fliF (A), flhB (B), and fliC (C) operons in ZW1, ZW1ΔTT, and ZW1ΔTT-adrA was measured by qRT-PCR with topA as an internal reference. For the convenience of comparison, the expression levels in ZW1 were normalized as 1. Data are presented as means ± SD, n = 3. Data marked with different letters have statistically significant differences (p < 0.05). (D) The motility of ZW1, ZW1ΔTT, and ZW1ΔTT-adrA on LB medium containing 0.3% (w/v) agar.

Figure 6. Thiamine uptake affects ETAE_1902 gene expression and bacterial adhesion through c-di-GMP. (A) The expression of ETAE_1902 in ZW1, ZW1ΔTT, and ZW1ΔTT-adrA was measured by qRT-PCR with topA as an internal reference. For the convenience of comparison, the expression levels in ZW1 were normalized as 1. (B) Turbot PBLs were incubated with ZW1, ZW1ΔTT, or ZW1ΔTT-adrA for 2 h, and the number of cell-attached bacteria was determined. Data are presented as means ± SD, n = 3. Data marked with different letters have statistically significant differences (p < 0.05).

3. Discussion
In this study we investigated the involvement of thiamine uptake in the virulence of E. piscicida. Thiamine is the precursor of TPP, which is an important coenzyme in central carbon metabolism and linked to virulence gene expression in intracellular bacteria [22,37]. In Xanthomonas oryzae, one of the TPP synthesis gene, thiG, was shown to be required for the full virulence of the bacterium [38]. In Pseudomonas aeruginosa, the deletion of thiL, which encodes an enzyme catalyzing the phosphorylation of thiamine monophosphate to produce TPP, resulted in markedly attenuated pathogenicity [39]. In V. dahliae, a thiamine transporter protein, VdThit, was important for bacterial invasion [25]. In L. monocytogenes, both thiamine uptake and the synthesis of thiamine precursors were essential to intracellular replication [26]. In E. piscicida, the genes for thiamine uptake and synthesis are present in the genome, however, their roles in pathogenicity are unknown. In this study, we
found that deletion of the thiBPQ thiamine transporter operon led to a markedly reduced intracellular level of TPP, indicating that *E. piscicida* acquires thiamine mainly through transportation from exogenous sources. Further, we discovered that the virulence of *E. piscicida* was significantly attenuated under the condition of reduced TPP. These results, together with the previous observations, suggest that thiamine, either by exogenous uptake or by biosynthesis, is possibly universally required for bacterial pathogenicity. However, the underlying mechanism remains to be explored.

In this study, we found that the swimming and host cell attachment abilities were obviously impaired in ZW1ΔTT. The genes whose expression may be responsible for the difference in these abilities were identified. For motility, they were *fliF-O*, *flhBAE*, and *fliC1C2AZ*, most of which are known to encode the structure proteins of the flagellar apparatus [40,41]. Notably, the *fliC* genes encoding flagellins, the flagellar filament structural proteins, have been reported to be essential for the virulence of *E. piscicida* [5]. For the genes associated with cellular adhesion, ETAE_1902 was found to be significantly repressed in expression upon TPP suppression. ETAE_1902 encodes a YadA-type adhesin. YadA is an autotransporter adhesin and a major virulence factor of *Y. enterocolitica* in which YadA mediates a bacterial attachment to the extracellular matrix components of eukaryotic host cells [42]. In our work, overexpression of ETAE_1902 restored the lost adhesion capacity of ZW1ΔTT, suggesting that YadA was likely a major thiamine-regulated factor in *E. piscicida* for host attachment.

In the present study, we conducted a search for the possible molecules and signaling routes linking thiamine availability to virulence traits in *E. piscicida*. We found that c-di-GMP synthesis was significantly repressed in the TPP-scarce mutant, thereby supporting the possibility that TPP might facilitate the biosynthesis of cyclic nucleotide second messengers by promoting the pentose phosphate pathway through transketolase [27]. C-di-GMP is known to play a role in several aspects of bacterial infection, including host cell adherence and invasion, bacterial motility, and the expression of virulence-associated genes [29,43]. For instance, in *L. monocytogenes* and *S. enterica*, the cellular invasion capacities are dampened by high concentrations of c-di-GMP [44,45]. In *Clostridium difficile*, the expression of the exotoxin genes *tcdA* and *tcdB* were repressed by c-di-GMP through a riboswitch-gated transcriptional activator [46]. In *P. aeruginosa*, the *cdrA* gene encoding a key adhesin in the exopolysaccharides (EPS), which participate in host cell adherence and biofilm formation, was transcriptionally induced in response to c-di-GMP elevation [29,47]. In *Borrelia burgdorferi*, the deletion of the gene coding for a c-di-GMP effector, PliZ, resulted in reduced bacterial motility and virulence [48]. In this work, we showed that c-di-GMP elevation induced by an exogenous DGC enzyme could restore the lost motility and host adhesion, as well as the associated gene expression, caused by TPP undersupply, therefore demonstrating the importance of c-di-GMP in the regulation of virulence associated with thiamine availability in *E. piscicida*. The possible mechanism underlying these observations may be as follows: the bacteria acquire exogenous thiamine via transportation to fuel the pentose phosphate pathway for the biosynthesis of c-di-GMP, which regulates virulence gene expression through effectors yet to be discovered and consequently affects the infective capacity of the bacteria, such as motility, host cell attachment, and presumably others, and eventually alters the bacterial pathogenicity (Figure 7).
Figure 7. Schematic diagram of the possible mechanism underlying the effects of thiamine uptake on Edwardsiella piscicida pathogenicity. Exogenous thiamine enters bacterial cells through the thiamine transporter and fuels the pentose phosphate pathway for the biosynthesis of c-di-GMP, which regulates the expression of virulence genes and consequently affects the virulence traits, such as motility and adhesion, leading eventually to altered bacterial pathogenicity.
4. Materials and Methods

4.1. Bacterial Strains and Growth Conditions

*E. piscicida* ZW1 was isolated from a diseased turbot from ZhuWang, Yantai, Shandong province, China in 2019. *E. coli* strains were from American Type Culture Collection (ATCC). All strains were grown in Luria–Bertani broth (LB) medium at 28 °C (for *E. piscicida*) or 37 °C (for *E. coli*). Where appropriate, polymyxin B, ampicillin, kanamycin, and chloramphenicol were supplemented at the concentrations of 50, 100, 50, and 30 µg/mL, respectively. The bacterial strains are listed in Supplementary Table S1.

4.2. Gene Deletion, Complementation, and Overexpression

For *thiBPQ* deletion, pDM4 was used for in-frame deletion as described previously [49]. In brief, fragments upstream and downstream of the *thiBPQ* operon were amplified and overlapped by PCR and then inserted to pDM4 at the indicated endonuclease sites (Table 1). The deletion mutant, ZW1ΔTT, was generated by two-step homologous recombination and verified by PCR and sequencing.

| Primer          | Sequence (5'-3')                           | Source        |
|-----------------|-------------------------------------------|---------------|
| TTF1            | ATCTCTAGACCTTATTTGAAACGCGCCAGC (XhoI)     | This study    |
| TTR1            | ATATGGCCGCTAGAAGACGCGCGTGTTGC             | This study    |
| TTF2            | TTCTGAGGCGCATATCGCTAGACGCAGG             | This study    |
| TTR2            | GCTGTCAGCTATCTCATAGCTAGTAACTCCA (SalI)   | This study    |
| TTAAGTTAATTAAAAGGGGCAGGA                  | This study    |
| TACAGATCTCTTACAGTATG                        | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TCGAGGGCGCGCAAGCTTTCTCGACCCTATG            | This study    |
| TCGTGACTCTCATACGGCACCCTGCTTGGCA (HindIII)  | This study    |
| TTF            | TACAGATCTCTTATTTGAAACGCGCCAGC (XhoI)     | This study    |
| TTR            | CCCATATACCTAGCGAACGCCAGGA                | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
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| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
| TACGCCAAAATCCCAGCTAGCTAGCAGC               | This study    |
Table 1. Cont.

| Primer       | Sequence (5′-3′)                                      | Source        |
|--------------|------------------------------------------------------|---------------|
| RT-fihA-R    | GATACCAGATGGCGGAAGTT                                  | This study    |
| RT-fihE-F    | GACTACCTCGTGCTGGCTAT                                  | This study    |
| RT-fihE-R    | GGCCTCAATAGGTTATCT                                   | This study    |
| RT-flgA-F    | CAGTGGCTTCATATGTCAG                                   | This study    |
| RT-flgA-R    | CTAAGAAACCAGCGTCAG                                    | This study    |
| RT-flgB-F    | CGCTTTCACCTCATCT                                       | This study    |
| RT-flgB-R    | ATAACGCCAGCTGTTGTC                                   | This study    |
| RT-fihD-F    | TTGTATATGACACAGCGATT                                  | This study    |
| RT-fihD-R    | CTGCCATCTACATTGATGG                                   | This study    |
| RT-filoc1-F  | TACCTGGCTGATATGACC                                    | This study    |
| RT-filoc1-R  | CTGAATAACATAGGCCAGAG                                  | This study    |
| RT-filoc2-F  | CTTCAACGCCAATATCAAC                                   | This study    |
| RT-filoc2-R  | GAGTTAGAGCGCTGCTG                                    | This study    |
| RT-filA-F    | GAAGGCCGTGATGACA                                       | This study    |
| RT-filA-R    | TGAATGGCAATGCTGTTGAA                                   | This study    |
| RT-filZ-F    | GAGATTGTGCGCTGCTG                                     | This study    |
| RT-filZ-R    | CGAAGCTGAAGATACATGCTGATA                               | This study    |
| RT-filD-F    | ACCACCAAGAGCATACATC                                   | This study    |
| RT-filD-R    | CTTACATATCTCATGCGCTTAA                                 | This study    |
| RT-filF-F    | CACGCCAATGAGATATC                                     | This study    |
| RT-filF-R    | ACCACCCAGATGGTGTGAT                                   | This study    |
| RT-filG-F    | CTGAGGTATATCTGCGAT                                    | This study    |
| RT-filG-R    | CAGGATGCTGGCGATGAT                                    | This study    |
| RT-filH-F    | GGGTTTTCAACAGGACGCT                                    | This study    |
| RT-filH-R    | GCCATGACATCTGAC                                       | This study    |
| RT-filI-F    | CATTAAACGCTGCCTGAC                                    | This study    |
| RT-filI-R    | CGCCGAGGATATCTC                                       | This study    |
| RT-filJ-F    | AGATGCTACTCAACTATCAAG                                  | This study    |
| RT-filJ-R    | CCAGCGTCAAGGATAAAACT                                  | This study    |
| RT-fik-F     | CCGTGGTCCGCGATATCT                                      | This study    |
| RT-fik-R     | CCCTGCTGATATCTGAG                                     | This study    |
| RT-filL-F    | AACGACATACATGCCGAT                                    | This study    |
| RT-filL-R    | TGAACGCTGGAACATAAC                                    | This study    |
| RT-film-F    | CAGCCGAATGAGATATC                                     | This study    |
| RT-film-R    | ACCGACACGATGATGAT                                     | This study    |
| RT-filN-F    | GTCAGAATAACGCGCATGAT                                  | This study    |
| RT-filN-R    | GGAATGGCCAGATGCA                                       | This study    |
| RT-fio-F     | AGGTGCAAAACTATGCT                                     | This study    |
| RT-fio-R     | CAGGACTTCTTATCTTCCG                                    | This study    |
|              | AGAAAGAAGATCCAAAAGATCTAAGAGAGA                       | This study    |
|              | GAAAGGATCTAGAAAAC                                       | This study    |
|              | CGTCATTCTATTTTACT                                     | This study    |
| 1902-OE-F    | GCCTGGAGATCTTCGAGTCAAGTAAAATTGGCGCC                    | This study    |
| 1902-OE-R    | TAGTTGACGCC                                      | This study    |
| 2842-OE-F    | AGAAGAAAGAATCAAAGATCTAAGAGAGA                        | This study    |
| 2842-OE-R    | GAAAGGATCTATGGGATTCGCTGTCCATTGCG                        | This study    |
| adfA-OE-F    | AGAAAGAAGAATCBAAGAAGGCTTACAGAGAGA                     | This study    |
| adfA-OE-R    | GCAAAAGTCTCCCAAGGCAACAGGAGAGA                         | This study    |

For the construction of the TT complement strain, the plasmid pCP1 was created by adding a MCS linker to pwtCas9-bacterial (Addgene 44250) at between the AflII and XhoI sites. The entire thiBPQ with the promoter region was amplified by PCR and inserted into
pCP1 at the indicated sites (Table 1). The resulting plasmid was introduced into ZW1∆TT by electroporation, yielding the complement strain ZW1∆TTc.

For gene overexpression, the PCR products of the indicated genes (adhesin/invasin genes from E. piscicida ZW1; adrA from E. coli MG1655) were inserted into pwtCas9-bacterial between the BglII and XhoI sites with the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China) to enable an inducible expression of these genes under a tetracycline promoter. For invasin/adhesin overexpression, the control plasmid pOE1c was constructed by blunting and ligating the BglII and XhoI digestion product of pwtCas9-bacterial. For adrA overexpression, the control plasmid pOE1-adrA* was constructed as reported previously [36], which expressed an inactivated AdrA. The resulting plasmids were introduced into indicated E. piscicida strains by electroporation. Where appropriate, the expression was induced by adding 1 µM anhydrotetracycline.

The TPP sensing plasmid pNmA was commercially synthesized with pEGFP-N2 as the bone vector. The plasmid was exactly the same as described in a previous publication [34], which contains a natural TPP riboswitch of serogroup A Neisseria meningitidis. In this plasmid, the TPP riboswitch forms a stem–loop structure in the presence of TPP, resembling a Rho-independent transcription terminator, thereby preventing transcription of the downstream gfp gene. pEGFP-N2 was used as the control plasmid. The plasmids were introduced into indicated E. piscicida strains by electroporation.

The c-di-GMP sensing plasmid pBc3–5 was commercially synthesized with pEGFP-N2 as the bone vector. This plasmid put a well-characterized c-di-GMP riboswitch [35] between a synthetic constitutive promoter J23100 and a strong RBS B0034, both were functionally verified and have frequently been documented as a combination for driving the expression of various genes such as gfp (BBa_K3740058 and others). The details and updates can be found at http://parts.igem.org (accessed on 2 March 2022). The above riboswitch-containing expression cassette was placed at the SmaI site of pEGFP-N2 and before the gfp gene. In the absence of c-di-GMP, the riboswitch terminates the transcription of the downstream gfp gene. pEGFP-N2 was used as the control plasmid. The plasmids were introduced into indicated E. piscicida strains by electroporation.

All primers are listed in Table 1.

4.3. Experimental Animals

Healthy Scophthalmus maximus (average 20 g or 500 g) were purchased from Hai-Shuo-Jia-Yuan aquaculture company in Qingdao city, Shandong province, China. The fish were maintained at 19 °C in aerated seawater and acclimatized in the laboratory for 2 weeks prior to experiments. Before experiments, fish were verified to be healthy by examination of potential bacterial existence in kidney and spleen as described previously [51].

4.4. In Vivo Infection

To compare the in vivo infectivity of E. piscicida ZW1, ZW1∆TT, and ZW1∆TTc, the abilities of these strains to disseminate in host tissues were examined. For this purpose, ZW1, ZW1∆TT, and ZW1∆TTc were cultured as above and collected at OD600 0.5. The bacteria were washed with and resuspended in PBS to a concentration of 2 × 10⁶ CFU/mL. Turbot (average 20 g) were randomly divided into three groups (9 fish/group). The fish of Group 1 (control group) were intramuscularly injected with 100 µL ZW1. The fish of Groups 2 and 3 were similarly injected with 100 µL ZW1∆TT and ZW1∆TTc, respectively. The dose of injection in all groups was 2 × 10⁵ CFU/fish. At 12 h, 24 h, and 48 h post-infection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma). The spleen and kidney were collected aseptically, homogenized, and serially diluted in PBS. The diluted homogenates were plated on LB agar and incubated at 28 °C for 24 h. The colonies were counted in number. To verify the identity of the bacteria, several clones were randomly picked for 16S rDNA sequencing by Tsingke, China. To examine the mortality-inducing capacity of the E. piscicida strains, turbot of two groups (10 fish/group) were injected
intramuscularly with ZW1 or ZW1ΔTT as described above. The fish were monitored daily for survival for 7 days.

4.5. Soft Agar Swimming Assay

*E. piscicida* ZW1, ZW1ΔTT, and ZW1ΔTTc were cultured as above and collected at OD_{600} 0.5. The bacteria were washed with and resuspended in PBS to a concentration of 1 × 10^8 CFU/mL. Ten-microliter of bacterial suspension was dropped onto LB plates containing 0.3% (w/v) agar. The plates were incubated at 28 °C for at least 2 days and observed for bacterial motility.

4.6. Cellular Adhesion Assay

Peripheral blood leukocytes (PBLs) were isolated from turbot (average 500 g) as reported previously [52] and adjusted to 10^7 cell/mL in L-15 medium (Gibico, Grand Island, NY, USA). *E. piscicida* strains were prepared as described above and suspended in L-15. PBLs were infected with *E. piscicida* at an MOI of 1:1 and incubated at 22 °C for 2 h with constant slow rotation. The cells were then lysed with 1% Triton X-100, and the lysate was serially diluted and plated on LB agar plates. The plates were incubated at 28 °C, and the colonies were counted and verified as above. To calculate the amount of internalized bacteria, the experiment was conducted as described as above, except that the extracellular and cell surface-attached bacteria were killed by treating with gentamycin (200 µg/mL) for 1 h before plate count.

4.7. qRT-PCR

Bacteria were prepared as described above, and 10^9 cells were lysed with Bacteria RNA Extraction Kit (Vazyme, Nanjing, China). RNA was extracted using Bacterial RNA Kit (Omega Bio-Tek, Guangzhou, China), with the optional on-membrane DNase I treatment step included to remove the residual genomic DNA. One microgram of purified RNA was used for reverse transcription with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed with three biological samples using the QuantStudio 3 Real-Time PCR System. Relative transcription was quantified by the comparative Ct (2^{-\Delta\Delta CT}) method with *topA* as an internal control [50]. For the comparison of flagellar and adhesion gene expression in ZW1, ZW1ΔTT, and ZW1ΔTTc, the empty plasmid pCP1 was introduced into both ZW1 and ZW1ΔTT. For the comparison of flagellar and adhesion gene expression in ZW1, ZW1ΔTT, and ZW1ΔTT-adrA, the control plasmid pOE1-adrA expressing an inactivated AdrA was introduced into ZW1 and ZW1ΔTT. In all cases, the gene expression level in ZW1 was set as 1.

All primers are listed in Table 1.

4.8. Fluorescence Intensity Quantification

Bacteria were prepared as above and resuspended in PBS. The suspension (200 µL) was transferred to a Costar® 3603 plate. The visible light absorption at 600 nm and the fluorescence intensity (FI) (excitation and emission wavelengths at 485 nm and 515 nm, respectively) were measured with a BioTek Synergy H1 multifunction reader (Winooski, VT, USA). The relative FI was calculated as the FI value per OD_{600}. The data were presented as the relative FI value of the reporter strain subtracted by the value of its control strain, which harbored the empty plasmid pEGFP-N2.

4.9. Statistical Analysis

All experiments were performed three times. Statistical analyses were carried out with the SPSS software (SPSS Inc., Chicago, IL, USA). Data were analyzed with Student’s t test or one-way ANOVA. Statistical significance was defined as *p* < 0.05.
5. Conclusions

In conclusion, the present work demonstrates that thiamine uptake abrogation imposed by deletion of the thibPQ-type thiamine transporter is able to create an intracellular TPP-scarce situation, which attenuates the overall pathogenicity of *E. piscicida*, and reduces host colonization and the expression of the associated virulence genes. TPP starvation leads to intracellular c-di-GMP suppression, probably through the pentose phosphate pathway. C-di-GMP supplementation restored the impaired virulence-associated gene expression and phenotypes caused by TPP reduction. These findings reveal an important role of thiamine uptake and c-di-GMP in the pathogenicity of *E. piscicida* and the participation of TPP in c-di-GMP signaling. Furthermore, by indicating an essentialness of TPP in bacterial virulence, our results also suggest that the TPP/thiamine acquisition pathway might be considered as the target for anti-*E. piscicida* drugs or therapies. C-di-GMP signaling and its role in the regulation of *E. piscicida* virulence require further investigation.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11040464/s1, Figure S1: Alignment of the sequences of ThiB, ThiP, and ThiQ; Figure S2: The survival rates of turbot infected with ZW1 and ZW1ΔTT; Figure S3: The effect of TT deletion on bacterial motility; Figure S4: The effects of TT deletion on the expression of flagellar genes; Figure S5: Bacteria recovery from turbot peripheral blood leukocytes (PBLs) infected with Edwardsiella piscicida strains; Figure S6: The effects of TT deletion on the expression of adhesion factor genes; Figure S7: The effect of adrA overexpression on bacterial motility; Table S1: Bacterial strains used in this study.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available in the article or supplementary material.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Leung, K.Y.; Wang, Q.Y.; Zheng, X.C.; Zhuang, M.; Yang, Z.Y.; Shao, S.; Achmon, Y.; Siame, B.A. Versatile lifestyles of *Edwardsiella*: Free-living, pathogen, and core bacterium of the aquatic resistome. *Virulence* 2022, 13, 5–18. [CrossRef] [PubMed]
2. Abayneh, T.; Colquhoun, D.J.; Sørum, H. *Edwardsiella piscicida* sp. nov. a novel species pathogenic to fish. *J. Appl. Microbiol.* 2013, 115, 634–635. [CrossRef] [PubMed]
3. Xu, T.T.; Zhang, X.-H. *Edwardsiella tarda*: An intriguing problem in aquaculture. *Aquaculture* 2014, 431, 129–135. [CrossRef]
4. Leung, K.Y.; Wang, Q.Y.; Yang, Z.Y.; Siame, B.A. *Edwardsiella piscicida*: A versatile emerging pathogen of fish. *Virulence* 2019, 10, 555–567. [CrossRef] [PubMed]
5. He, Y.; Xu, T.T.; Fossheim, L.E.; Zhang, X.-H. FlIC, a Flagellin Protein, Is Essential for the Growth and Virulence of Fish Pathogen *Edwardsiella tarda*. *PLoS ONE* 2012, 7, e45070. [CrossRef]
6. Sun, Y.; Zheng, W.-J.; Hu, Y.-H.; Sun, B.-G.; Sun, L. *Edwardsiella tarda* Eta1, an in vivo-Induced Antigen that is Involved in Host Infection. *Infect. Immun.* 2012, 80, 2948–2955. [CrossRef]
7. Li, M.F.; Hu, Y.H.; Zheng, W.J.; Sun, B.G.; Wang, C.L.; Sun, L. Inv1: An *Edwardsiella tarda* invasin and a protective immunogen that is required for host infection. *Fish Shellfish Immunol.* 2012, 32, 586–592. [CrossRef]
8. Zhou, Z.-J.; Sun, B.-G.; Sun, L. *Edwardsiella tarda* Sip1: A serum-induced zinc metalloprotease that is essential to serum resistance and host infection. *Vet. Microbiol.* 2015, 177, 332–340. [CrossRef]
9. Wang, B.; Mo, Z.L.; Xiao, P.; Li, J.; Zou, Y.X.; Hao, B.; Li, G.Y. EseD, a Putative T3SS Translocon Component of *Edwardsiella tarda*, Contributes to Virulence in Fish and is a Candidate for Vaccine Development. *Mar. Biotechnol.* 2010, 12, 678–685. [CrossRef]
10. Wang, B.; Mo, Z.L.; Mao, Y.X.; Zou, Y.X.; Xiao, P.; Li, J.; Yang, J.Y.; Ye, X.H.; Leung, K.Y.; Zhang, P.J. Investigation of EscA as a chaperone for the Edwardsiella tarda type III secretion system putative translocon component EseC. *Microbiol. Res.* 2009, 155 Pt 4, 1260–1271. [CrossRef]

11. Chen, H.; Yang, D.; Han, F.; Tan, J.; Zhang, L.; Xiao, J.; Zhang, Y.; Liu, Q. The Bacterial T6SS Effector EvpP Prevents NLRR3 Inflammasome Activation by Inhibiting the Ca^{2+}-Dependent MAPK-Ink Pathway. *Cell Host Microbe* 2017, 21, 47–58. [CrossRef] [PubMed]

12. Buján, N.; Toranzo, A.E.; Magarinos, B. Edwardsiella piscicida: A significant bacterial pathogen of cultured fish. *Dis. Aquat. Org.* 2018, 131, 59–71. [CrossRef]

13. Li, D.Y.; Liu, Y.L.; Liao, X.J.; He, T.T.; Sun, S.S.; Nie, P.; Xie, H.X. Identification and Characterization of EvpQ, a Novel T6SS Effector Encoded on a Mobile Genetic Element in *Edwardsiella piscicida*. *Front. Microbiol.* 2021, 12, 643948. [CrossRef]

14. Leung, K.Y.; Siame, B.A.; Tenkink, B.J.; Noort, R.J.; Mok, Y.-K. *Edwardsiella tarda*—Virulence mechanisms of an emerging gastroenteritis pathogen. *Microbes Infect.* 2012, 14, 26–34. [CrossRef] [PubMed]

15. Ling, S.H.M.; Wang, X.H.; Lim, T.M.; Leung, K.Y. Green fluorescent protein-tagged *Edwardsiella tarda* reveals portal of entry in fish. *Fems. Microbiol. Lett.* 2001, 201, 239–243. [CrossRef]

16. Wei, L.; Qiao, H.; Sit, B.; Yin, K.; Yang, G.; Ma, R.; Ma, J.; Yang, C.; Yao, J.; Ma; Y.; et al. A Bacterial Pathogen Senses Host Mannose to Coordinate Virulence. *Science* 2019, 36, 310–323. [CrossRef]

17. Yin, K.; Zhang, J.; Ma, J.; Jin, P.; Ma, Y.; Zhang, Y.; Liu, X.; Wang, Q. MviN mediates the regulation of environmental osmotic pressure on esrB to control the virulence in the marine fish pathogen *Edwardsiella piscicida*. *Microbiol. Res.* 2020, 239, 126528. [CrossRef] [PubMed]

18. Jurgenson, C.T.; Begley, T.P.; Ealick, S.E. The Structural and Biochemical Foundations of Thiamin Biosynthesis. *Annu. Rev. Biochem.* 2009, 78, 569–603. [CrossRef] [PubMed]

19. Zhang, K.; Bian, J.; Deng, Y.; Smith, A.; Nunez, R.E.; Li, M.B.; Pal, U.; Yu, A.-M.; Qiu, W.; Ealick, S.E.; et al. Lyme disease spirochaete *Borreli burgdorferi* does not require thiamin. *Nat. Microbiol.* 2016, 2, 16213. [CrossRef]

20. Aleshin, V.A.; Mkrtchyan, G.V.; Derewenda, Z.S. The Structure and Ligand Binding Properties of Thiamin/HMP-binding Proteins. *Biochemistry* 2014, 53, 4485–4493. [CrossRef] [PubMed]

21. Buján, N.; Toranzo, A.E.; Magarinos, B. Edwardsiella piscicida: A significant bacterial pathogen of cultured fish. *Dis. Aquat. Org.* 2018, 131, 59–71. [CrossRef]

22. Begley, T.P.; Downs, D.M.; Ealick, S.E.; McLafferty, F.W.; Van Loon, A.P.G.M.; Taylor, S.; Campobasso, N.; Chiu, H.-J.; Kinsland, C.; et al. Vitamin B1 (Thiamine) Metabolism and Regulation in Archaea. In *Annu. Rev. Biochem.* 2019, 88, 829–850. [CrossRef]

23. Webb, E.; Claas, K.; Downs, D. thlBFQ encodes an ABC transporter required for transport of thiamine and thiamine pyrophosphate in *Salmonella typhimurium*. *J. Mol. Biol.* 2016, 343, 395–406. [CrossRef] [PubMed]

24. Wang, Q.; Yang, M.; Xiao, J.; Wu, H.; Wang, X.; Lv, Y.; Xu, L.; Zheng, H.; Wang, S.; Zhao, G.; et al. Genome sequence of the *Edwardsiella piscicida* ESEC strain T3 represents an emerging fish pathogen. *Appl. Environ. Microbiol.* 2016, 82, 4485–4493. [CrossRef] [PubMed]

25. Wang, Q.; Yang, M.; Xiao, J.; Wu, H.; Wang, X.; Lv, Y.; Xu, L.; Zheng, H.; Wang, S.; Zhao, G.; et al. Genome sequence of the *Edwardsiella piscicida* ESEC strain T3 provides insights into its adaptation to broad host ranges and intracellular niches. *PLoS ONE* 2019, 4, e7646. [CrossRef] [PubMed]

26. Römling, U.; Galperin, M.Y.; Gomelsky, M. Cyclic di-GMP: The First 25 Years of a Universal Bacterial Second Messenger. *Microbiol. Res.* 2013, 177, 51–72. [CrossRef]

27. Römling, U.; Galperin, M.Y.; Gomelsky, M. Cyclic di-GMP: The First 25 Years of a Universal Bacterial Second Messenger. *Microbiol. Res.* 2013, 177, 51–72. [CrossRef]

28. Schauer, K.; Stolz, J.; Scherer, S.; Fuchs, T.M. Both Thiamine Uptake and Biosynthesis of Thiamine Precursors are Required for Intracellular Replication of *Listeria monocytogenes*. *J. Bacteriol.* 2009, 191, 2218–2227. [CrossRef] [PubMed]

29. Tylicki, A.; Łotowski, Z.; Siemieniuk, M.; Ratkiewicz, A.; Thiamine and selected thiamine antivitamins—Biological activity and methods of synthesis. *Biosci. Rep.* 2018, 38, 38. [CrossRef] [PubMed]

30. Hengge, R. Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* 2009, 7, 263–273. [CrossRef]

31. Chen, H.; Yang, D.; Han, F.; Tan, J.; Zhang, L.; Xiao, J.; Zhang, Y.; Liu, Q.; Ye, X.H.; Leung, K.Y. Investigation of EscA as a chaperone for the Edwardsiella tarda type III secretion system putative translocon component EseC. *Microbiol. Res.* 2009, 155 Pt 4, 1260–1271. [CrossRef]

32. Srivastava, D.; Waters, C.M. A Tangled Web: Regulatory Connections between Quorum Sensing and Cyclic Di-GMP. *J. Bacteriol.* 2012, 194, 4485–4493. [CrossRef] [PubMed]

33. Wang, Q.; Yang, M.; Xiao, J.; Wu, H.; Wang, X.; Lv, Y.; Xu, L.; Zheng, H.; Wang, S.; Zhao, G.; et al. Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. *PLoS ONE* 2019, 4, e7646. [CrossRef] [PubMed]

34. Wang, Q.; Yang, M.; Xiao, J.; Wu, H.; Wang, X.; Lv, Y.; Xu, L.; Zheng, H.; Wang, S.; Zhao, G.; et al. Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. *PLoS ONE* 2019, 4, e7646. [CrossRef] [PubMed]

35. Zhou, H.; Zheng, C.; Su, J.; Chen, B.; Fu, Y.; Xie, Y.; Tang, Q.; Chou, S.-H.; He, J. Characterization of a natural triple-tandem c-di-GMP riboswitch and application of the riboswitch-based dual-fluorescence reporter. *Sci. Rep.* 2016, 6, 20871. [CrossRef] [PubMed]

36. Antonyan, D.; Bocci, P.; Maciag, A.; Raffaelli, N.; Landini, P. Monitoring of diguanulate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. *Appl. Microbiol. Biotechnol.* 2010, 85, 1095–1104. [CrossRef] [PubMed]
37. Eisenreich, W.; Dandekar, T.; Heesemann, J.; Goebel, W. Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. Nat. Rev. Microbiol. 2010, 8, 401–412. [CrossRef] [PubMed]

38. Yu, X.; Liang, X.; Liu, K.; Dong, W.; Wang, J.; Zhou, M.-G. The thiG Gene Is Required for Full Virulence of Xanthomonas oryzae pv. oryzae by Preventing Cell Aggregation. PLoS ONE 2015, 10, e0134237. [CrossRef]

39. Kim, H.J.; Lee, H.; Lee, Y.; Choi, I.; Ko, Y.; Lee, S.; Jang, S. The ThiL enzyme is a valid antibacterial target essential for both thiamine biosynthesis and salvage pathways in Pseudomonas aeruginosa. J. Biol. Chem. 2020, 295, 10081–10091. [CrossRef]

40. Morimoto, Y.V.; Ito, M.; Hiraoka, K.D.; Che, Y.S.; Bai, F.; Kami-Ike, N.; Namba, K.; Minamino, T. Assembly and stoichiometry of Flf and FlhA in Salmonella flagellar basal body. Mol. Microbiol. 2014, 91, 1214–1226. [CrossRef]

41. Van Gerven, N.; Waksman, G.; Remaut, H. Pili and flagella biology, structure, and biotechnological applications. Prog. Mol. Biol. Transl. Sci. 2011, 103, 21–72.

42. Muhlenkamp, M.; Oberhettinger, P.; Leo, J.C.; Linke, D.; Schutz, M.S. Yersinia adhesin A (YadA)—Beauty & beast. Int. J. Med. Microbiol. 2015, 305, 21–72. [PubMed]

43. Valentini, M.; Filloux, A. Multiple Roles of c-di-GMP Signaling in Bacterial Pathogenesis. Annu. Rev. Microbiol. 2019, 73, 387–406. [CrossRef] [PubMed]

44. Chen, L.-H.; Köseoğlu, V.K.; Güvener, Z.T.; Myers-Morales, T.; Reed, J.M.; D’Orazio, S.E.F.; Miller, K.W.; Gomelsky, M. Cyclic di-GMP-dependent Signaling Pathways in the Pathogenic Firmicute Listeria monocytogenes. PLOS Pathog. 2014, 10, e1004301. [CrossRef] [PubMed]

45. Lamprokostopoulou, A.; Monteiro, C.; Rhen, M.; Romling, U. Cyclic di-GMP signalling controls virulence properties of Salmonella enterica serovar Typhimurium at the mucosal lining. Environ. Microbiol. 2010, 12, 40–53. [CrossRef] [PubMed]

46. McKee, R.W.; Mangalea, M.R.; Purcell, E.B.; Borchardt, E.K.; Tamayo, R. The Second Messenger Cyclic Di-GMP Regulates Clostridium difficile Toxin Production by Controlling Expression of sigD. J. Bacteriol. 2013, 195, 5174–5185. [CrossRef]

47. Borlee, B.R.; Goldman, A.D.; Murakami, K.; Samudrala, R.; Wozniak, D.J.; Parsek, M.R. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol. Microbiol. 2010, 75, 827–842. [CrossRef]

48. Fitz, J.E.; Sultan, S.Z.; Hayakawa, Y.; Hobbs, G.; Miller, M.R.; Motaleb, M.A. Analysis of the Borrelia burgdorferi cyclic-di-GMP-binding protein PlzA reveals a role in motility and virulence. Infect. Immun. 2011, 79, 1815–1825. [CrossRef]

49. Milton, D.L.; O’Toole, R.; Horstedt, P.; Wolt-Watz, H. Flagellin A is essential for the virulence of Vibrio anguillarum. J. Bacteriol. 1996, 178, 1310–1319. [CrossRef]

50. Sun, Z.; Deng, J.; Wu, H.; Wang, Q.; Zhang, Y. Selection of Stable Reference Genes for Real-Time Quantitative PCR Analysis in Edwardsiella tarda. J. Microbiol. Biotechnol. 2017, 27, 112–121. [CrossRef]

51. Zhou, Z.-X.; Zhang, B.-C.; Sun, L. Poly(I:C) Induces Antiviral Immune Responses in Japanese Flounder (Paralichthys olivaceus) that Require TLR3 and MDA5 and is Negatively Regulated by Myd88. PLoS ONE 2014, 9, e112918. [CrossRef]

52. Hu, Y.-H.; Chen, L.; Sun, L. CXCL8 of Scophthalmus maximus: Expression, biological activity and immunoregulatory effect. Dev. Comp. Immunol. 2011, 35, 1032–1039. [CrossRef] [PubMed]