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Impact of flaH Gene Mutations on Aeromonas caviae Swarming Motility: An Experimental study

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ABSTRACT

Background: Aeromonas caviae is an opportunistic pathogen that utilizes both polar and lateral flagella for motility. The flaH gene is believed to be essential for flagellar assembly and motility, particularly swarming. Methods: A flaH mutant strain (A. caviae AAR59) was generated via insertion of a kanamycin resistance cassette. Motility assays and genetic complementation were performed. Recombinant flaH was cloned, expressed, and purified for characterization. Results: Mutation of flaH abolished motility and flagellin expression. Complementation partially restored motility. Overexpressed flaH protein was soluble, suggesting its role in flagellar structure and function. Conclusion: flaH is critical for A. caviae motility and flagellar biogenesis. These findings enhance our understanding of bacterial motility and suggest flaH as a potential therapeutic target.

KEYWORDS: Aeromonas caviae, flaH gene, swarming motility, flagellar assembly, gene mutation.

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INTRODUCTION

Bacterial motility is a fundamental trait that influences colonization, biofilm formation, and pathogenesis. *Aeromonas caviae*, a Gram-negative facultative anaerobe, is an opportunistic pathogen frequently associated with gastrointestinal and extra-intestinal infections in humans (Majeed, De Silva et al. 2023, Wang, Zhao et al. 2023). A critical virulence factor of *A. caviae* is its flagellar system, which facilitates two distinct types of motilities: swimming, mediated by a single polar flagellum in liquid environments, and swarming, facilitated by multiple lateral flagella on solid surfaces (Mendoza-Barberá, Merino et al. 2021, Qu and Liu 2024). Swarming motility is a highly coordinated, multicellular behavior that plays an essential role in bacterial colonization, biofilm formation, and host-pathogen interactions (Nedeljković, Sastre et al. 2021, Chakkour, Hammoud et al. 2024, Somaili, Oraibia et al. 2024). The structural integrity and function of the flagellar apparatus are regulated by multiple genes, among which *flaH* has been implicated in flagellin glycosylation, stability, and assembly (Al-Otaibi and Bergeron 2022, Fulton 2024, Unay, Kint et al. 2024). While the flagellin glycosylation has been extensively studied in *Campylobacter* and *Helicobacter* species, its precise role in *A. caviae* remains poorly understood (Fulton, Rosenthal et al. 2021, Lowry, Allihaybi et al. 2022). It is hypothesized that glycosylation enhances flagellar filament stability, bacterial adhesion, and immune evasion, thereby influencing motility and virulence(Akahoshi and Bevins 2022).

Previous studies have suggested that the *flm* gene cluster, which shares homology with genes involved in lipopolysaccharide (LPS) biosynthesis and protein glycosylation, is critical for both O-antigen adherence and flagella-mediated motility (Mendoza-Barberá, Merino et al. 2021, Manner 2023). Specifically, *A. caviae* Sch3 possesses the smallest known set of glycosylation genes required for pseudaminic acid biosynthesis and its incorporation onto flagellins, highlighting its potential as a model organism for studying flagellar glycosylation (Khairnar, Sunsunwal et al. 2021, Sunsunwal, Khairnar et al. 2024).

This study aims to investigate the impact of *flaH* gene mutations on *A. caviae* swarming motility by employing molecular genetics, mutagenesis, and phenotypic assays. By elucidating the role of *flaH* in flagellar assembly and function, this research will provide novel insights into bacterial motility mechanisms and their implications for pathogenicity. The other goal is to determine whether *flaH* genes can complement the analogous mutants in *A. caviae*.

MATERIALS AND METHODS

Bacterial strains, Plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in Luria–Bertani (LB) broth or LB Miller agar, whereas *A. caviae* strains were grown in brain heart infusion broth (BHIB) (Oxoid, Basingstoke, UK). Unless otherwise specified, cultures were incubated at 37°C under aerobic conditions. When required, antibiotics were added at the following concentrations: Ampicillin (50 µg/mL), Nalidixic acid (50 µg/mL), Kanamycin (50 µg/mL), Streptomycin (50 µg/mL), and Chloramphenicol (25 µg/mL).

Table 1. Bacterial strains and Plasmids used in this study

Genotype and Description	Strain or Plasmid	Reference
E. coli overexpression vector for N-terminal His_6-tagged proteins, Kmr	pET-28a(+)	Novagen
Broad-host-range vector, IncP, -W, -Q, ColE1, and p15A compatible, Cmr	pBBR1MCS	Kovach et al. (1994)
E. coli cloning strain **Φ80dlacZ ΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+) phoA supE44 λ-thi-1*	*E. coli* DH5α	Hanahan (1993)
E. coli F^- ompT hsdSB(rB^- mB^-) gal dcm (DE3) used for recombinant protein expression	BL21(DE3)	Laboratory stock
E. coli conjugation donor strain **hsdR, pro, recA, RP4-2 in chromosome Km::Tn7 (Tc::Mu) lpir, Tpr Smr*	S17λ-pir	de Lorenzo et al. (1990)
Wild-type *Aeromonas caviae* strain	*A. caviae* Sch3	J.G. Shaw lab
A. caviae mutant strain flaH::Kmr	AAR59 flaH::Kmr	Previous study

Abbreviations: r = resistance; Amp = ampicillin; Cm = chloramphenicol; Sm = streptomycin; Km = kanamycin; Nal = nalidixic acid; MCS = multiple cloning site.

PCR and primer design

The *flaH* gene was amplified using gene-specific primers, as shown in Table 2. PCR reactions were performed using Pfu DNA polymerase (Stratagene) in a reaction mixture containing 2.5 mM MgCl. PCR amplification was carried out in a Hybaid Omnigene Thermal Cycler.

The cycling conditions included an initial denaturation step at 94°C for 2 minutes, followed by 10 cycles of denaturation at 94°C for 15 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute. This was followed by an additional 20 cycles with a slightly increased annealing temperature of 52°C for 30 seconds and an extension time of 1 minute and 20 seconds. A final extension was performed at 72°C for 4 minutes, after which the samples were held at 4°C.

Table 2. Primers used in this study

Sequence	Restriction Site	Primer Name	Usage
ATATATCATATGGCCAATGAAATGATAGCC	NdeI (CATATG)	JLP_153	flaH - pET28 Forward
TATTATGGATCCTCACACTAGGCCATCGAAC	BamHI (GGATCC)	JLP_154	flaH - pET28 Reverse
5'-TCACACAGGAAACAGCTATGAC-3'	—	M13 Primers	General sequencing

Agarose gel electrophoresis and DNA purification

PCR-amplified DNA was analyzed via 1% agarose gel electrophoresis using 1× TAE buffer. The gel was prepared by dissolving 1g of agarose in 100 mL of 1× TAE buffer, followed by microwaving until completely dissolved. Ethidium bromide (0.5 µg/mL) was added to visualize DNA bands under UV illumination (240–366 nm). The DNA was purified using a Qiagen PCR purification kit, following the manufacturer's protocol.

Cloning and expression of His-tagged flaH

The amplified flaH fragment was cloned into the pET-28a(+) expression vector using NdeI and BamHI restriction sites, generating an N-terminal His_6-tagged fusion protein. The recombinant plasmid (pET28-flaH) was transformed into *E. coli* DH5α, then sequenced and further transformed into BL21(DE3) for protein expression. For heterologous expression in *A. caviae,* pET28-flaH was digested with BamHI and BglII, gel-extracted, and ligated to pbbr-mcs1(cm–R), a broad-host-range vector.

Bacterial conjugation

Successful constructs were transferred from *E. coli* S17λ-pir to W.T-A. *caviae* Sch3N strains via conjugation. Donor and recipient cultures were centrifuged (4,500 × g, 10 min, RT), washed, and resuspended in PBS. Equal volumes were mixed and spotted onto nitrocellulose filters on blood agar plates and incubated at 30°C for 6 h. Bacteria were recovered and plated on LB agar with kanamycin and chloramphenicol to select for transconjugants.

Motility and swarming assays

Motility agar plates (0.3% agar) were prepared with 1% tryptone and 0.5% NaCl. Bacterial cultures were inoculated at the center of the agar and incubated at 37°C for 16–18 h. Swarming motility was assessed by measuring the migration diameter.

Statistical analysis

Differences between wild-type, mutant, and complemented strains were analyzed using GraphPad Prism 8.0. Data are presented as mean ± standard deviation (SD). One-way ANOVA was used to assess significance, with $p < 0.05$ considered statistically significant.

RESULTS

Mutation of *flaH* abolishes motility in *Aeromonas caviae*

To investigate the role of the *flaH* gene in *Aeromonas caviae* motility, we utilized the AAR59 mutant strain, previously generated by insertion of a kanamycin resistance cassette into *flaH* in the same transcriptional orientation. Motility assays using semi-solid agar revealed a complete loss of motility in the mutant strain compared to the wild-type control (Figure 1). This result highlights the essential role of *flaH* in flagellar assembly and function.

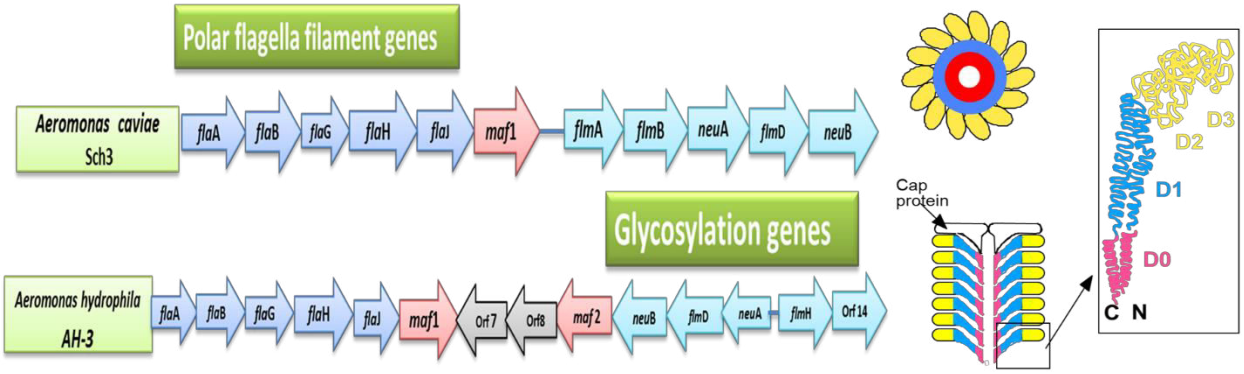

Figure 1: Genetic organization of *Aeromonas caviae* Sch3 and *Aeromonas hydrophila* AH-3 flagellar filament and flagellar glycosylation loci. ORFs are named after homologues in other bacteria. Arrows indicate direction of transcription. R.M. Macnab in the Journal of Bacteriology in 1999[i]

Genetic complementation partially restores motility

To confirm the functional role of *flaH*, complementation was performed by introducing the gene in trans using the broad-host-range plasmid pBBR1MCS-1. The *flaH* gene was cloned and inserted into the vector, followed by transformation into *E. coli* DH5α for screening and verification via M13 PCR primers. Correct constructs were sequenced and then transferred to *E. coli* S17λ-pir for conjugation with *A. caviae* Sch3N. Transconjugants were selected and evaluated for motility. Swarming motility assays on 0.3% agar indicated partial restoration of motility in complemented strains (Figure 2A). Quantitative measurement revealed a mean halo diameter of 20.8 mm for the complemented mutant versus 13.6 mm for the wild type—a 1.5-fold increase ($p < 0.0001$), suggesting partial but significant functional recovery (Figure 2B).

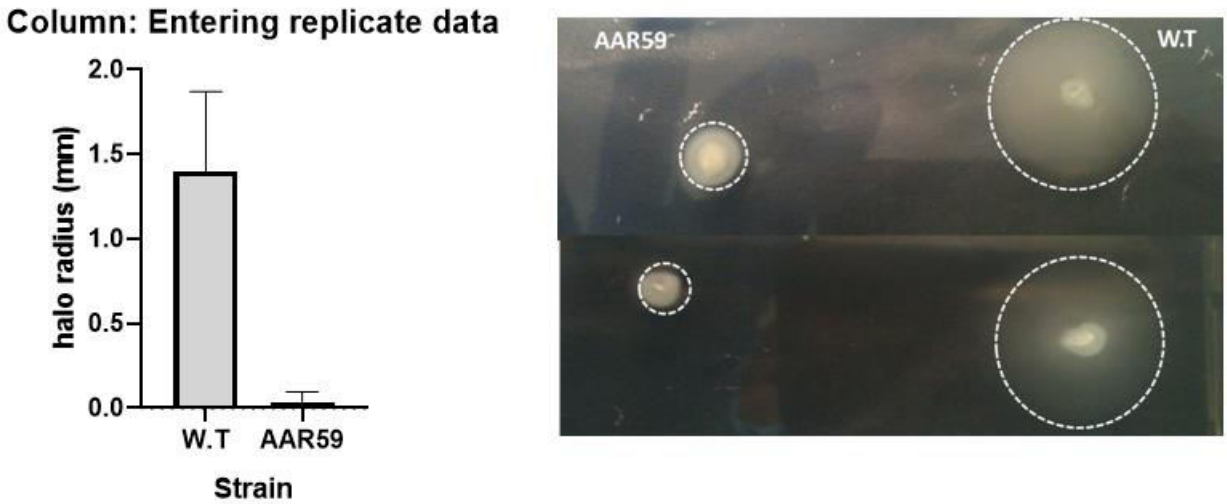

Figure 2. Loss of motility and flagellin expression in *A. caviae* AAR59 mutant. The *flaH* mutation led to a non-motile phenotype with an absence of visible flagellar structures.

Interestingly, the AHA0618 mutant exhibited hypermotility on swarming agar. However, due to irregular colony morphology, precise quantification was not feasible (Figure 2C-E).

***flaH* encodes a soluble protein**

To evaluate the biochemical nature of *flaH*, the gene was cloned into the expression vector pET28a(+), incorporating an N-terminal His_6-tag. The construct was confirmed via restriction digestion and sequencing, and then transformed into *E. coli* BL21 C41(DE3) for overexpression.

Agarose gel electrophoresis confirmed the expected 1.4 kb insert size (Figure 3). SDS-PAGE of induced cultures demonstrated soluble expression of the His-tagged FlaH protein, which was subsequently purified for antibody generation and downstream applications such as Western blotting and protein-protein interaction studies (Figure 4).

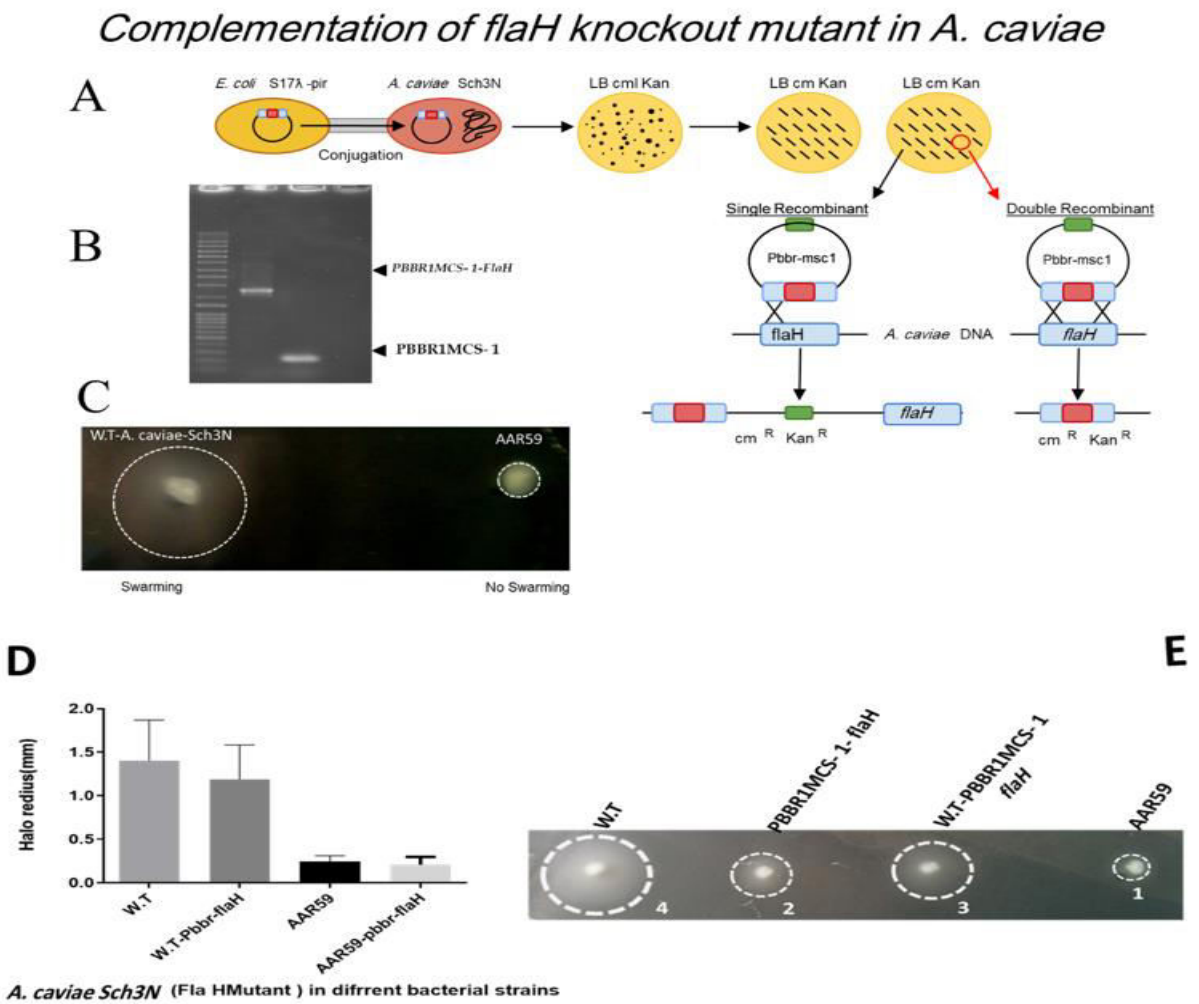

Figure 3. Schematic of *flaH* gene complementation strategy. The diagram outlines the construction of pBBR1MCS-1-*flaH* and subsequent conjugation into the *A. caviae* AAR59 mutant. Swarming assay results. A: Wild-type and mutant motility zones on 0.3% agar. B: Motility halo measurements showing significant increase in complemented strains (p< 0.0001). C: Irregular swarming patterns in hypermotile AHA0618 strain. D and E: flaH mutant and their halo measurements.

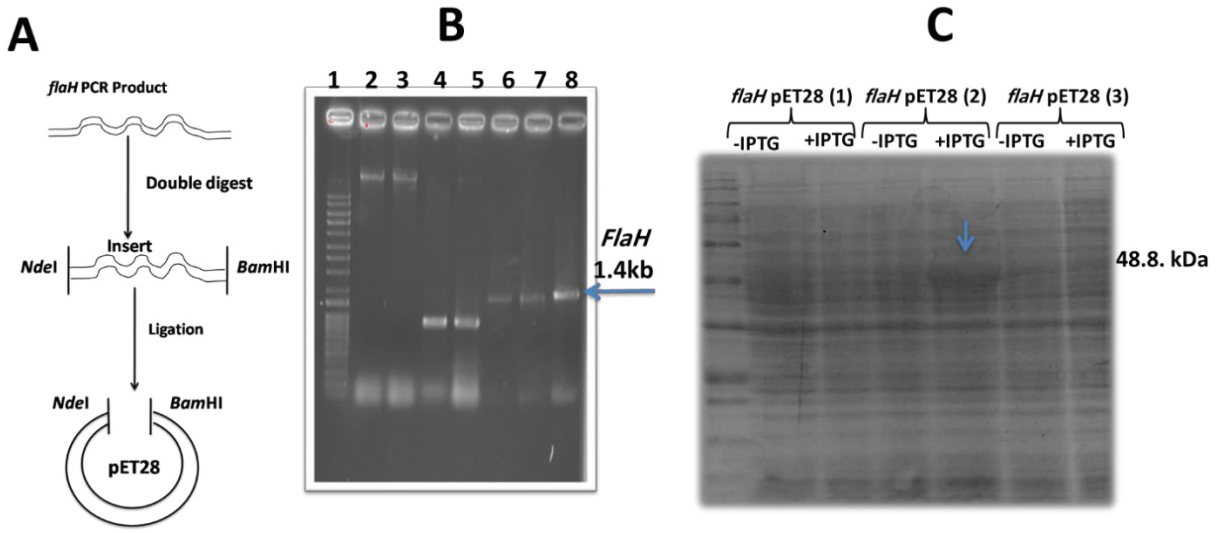

Figure 4. Molecular cloning of *flaH* into pET28a(+). Lane 6–8: Positive transformants containing the expected insert size after restriction enzyme digestion.

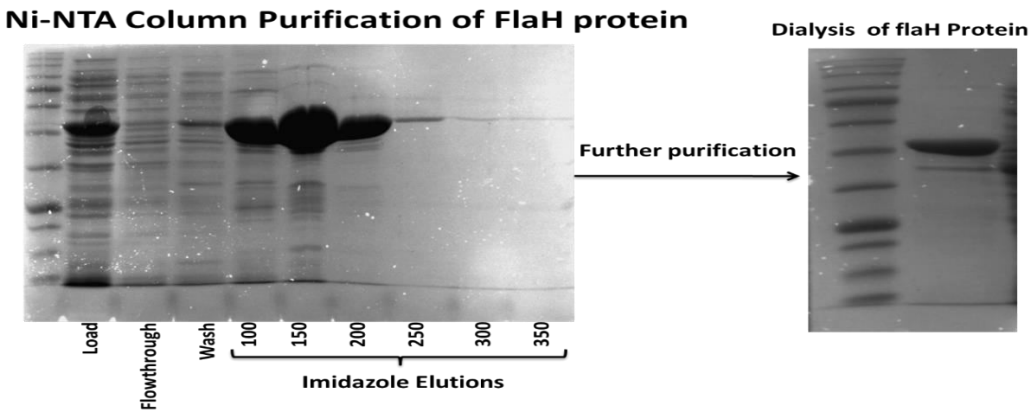

Figure 5. Expression and solubility of His$_6$-FlaH. SDS-PAGE image showing successful induction of soluble recombinant protein in BL21 C41(DE3) cells.

DISCUSSION

This study demonstrates that the *flaH* gene is critical for the flagellar biosynthesis and motility of *Aeromonas caviae*, a Gram-negative pathogen known for its dual flagellar systems. The flaH mutation led to complete loss of motility and absence of flagellin production, highlighting the gene's indispensable role in flagellar apparatus assembly. This aligns with previous observations in other species such as *Campylobacter jejuni* and *Pseudomonas aeruginosa*, where flagellar glycosylation genes similarly influence motility and virulence(Mendoza-Barberá, Merino et al. 2021, Lowry, Allihaybi et al. 2022) .

Complementation of the flaH mutant partially restored motility, though the recovery did not reach wild-type levels. This partial restoration could be attributed to the overexpression or misregulation of flaH when carried on a multicopy plasmid, which may disrupt the stoichiometric balance necessary for efficient flagellar assembly(Fulton, Zimmerman et al. 2024, Schwieters and Ahmer 2024).Similar effects have been observed in other bacterial systems, where gene dosage and regulatory context significantly influence motility phenotypes(Stabryla, Johnston et al. 2021) .

Interestingly, the emergence of hypermotility in the AHA0618 strain suggests that altered expression of flaH or associated regulatory elements may enhance swarming behavior under certain conditions. This phenomenon warrants further investigation into the regulation of lateral flagellar gene clusters and their interaction with surface sensing pathways (Matilla, Velando et al. 2022).

In addition to confirming flaH's role in polar flagella biosynthesis, this study also showed that the FlaH protein is soluble and can be expressed in *E. coli*, making it a suitable candidate for antibody production and protein–protein interaction studies. Such tools will be valuable for exploring the molecular partners of FlaH and its precise role in flagellar cap formation or chaperone activity(Mendoza-Barberá, Merino et al. 2021, Zhang, Ye et al. 2024).

The implications of these findings extend beyond basic bacterial physiology. Flagellar motility is directly linked to host colonization, immune evasion, and biofilm formation in *Aeromonas* spp.(Lau, Puah et al. 2023, Tao, Wang et al. 2024). Disruption of motility may serve as a viable therapeutic approach to attenuate virulence and reduce infection severity, especially given the increasing antimicrobial resistance reported among clinical isolates of *A. caviae* (Song, Wang et al. 2023, Ansari and Nagar 2024). Future work should aim to delineate the regulatory networks that coordinate flaH expression, glycosylation machinery. Advanced transcriptomic and proteomic profiling investigate the other proteins encoded in the flagellar filament operon that are also exported like the flagellins (FlaH) are also glycosylated.

CONCLUSION

Aeromonas caviae motility is dependent on the flaH gene, which is essential for flagellar biosynthesis and function. The flaH mutant strain (AAR59) exhibited a complete loss of motility due to the disruption of flagellin production and assembly. Complemented strains partially recovered motility, but the results emphasized the importance of proper gene regulation and expression balance. These findings advance our understanding of the genetic control of bacterial locomotion and highlight flaH as a promising target for developing anti-motility therapeutics against Aeromonas infections. Future studies should explore the regulatory pathways and potential interactions of flaH with other flagellar and glycosylation-associated genes to develop a more comprehensive picture of its role in motility and pathogenesis.

Statements and Declarations

Ethical Approval: This study did not involve human participants, human data, or human tissue. Therefore, ethical approval and informed consent were not required.

Consent for publication: Not applicable. This study did not involve human participants or any individual data requiring consent for publication.

Availability of data and material: The data are available from the corresponding author on reasonable request.

Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

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Author Contributions: The author conceived the study, performed the research, analyzed the data, and wrote the manuscript. The author read and approved the final manuscript.

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