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Original Paper

Isolation and characterization of gill mucus-associated antagonistic bacteria in the Asian swamp eel (*Monopterus albus*)

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Abstract

This study investigated the gill mucus of Asian swamp eel (*Monopterus albus*) for the presence of antagonistic bacteria against known aquatic pathogens, *Vibrio harveyi* and *Aeromonas hydrophila*. Initial screening of 500 bacterial isolates via the spot-on-lawn and co-incubation assays, identified five with significant antagonistic activity, which were further subjected to morphological, enzymatic, biochemical, and molecular characterization. All five isolates were Gram-negative bacilli. Four isolates were identified as belonging to the *Pseudomonas* genus, a known probiont with documented biocontrol properties in various plant and animal species. The remaining isolate exhibited high 16S rRNA gene sequence similarity to *Aeromonas dhakensis*, a known human pathogen. All isolates demonstrated the ability to produce at least three of six tested extracellular enzymes: catalase, amylase, protease, lipase, gelatinase, and urease. Given the observed antagonistic activity of these isolates, further research is warranted to evaluate their potential application as probiotics. This study represents the first investigation of antagonistic bacteria in swamp eel gill mucus and contributes to the limited research on fish gill mucus as a source of such bacteria.

Key words: antagonism, aquaculture, fish health, mucosal immunity, pathogens.
Abbreviations: CFU, colony forming units; NA, Nutrient Agar; NB, Nutrient Broth.

Introduction

The mucosa has an integral role in the fish innate immune system as the frontline defense against environmental and biological factors that might make the host susceptible to disease (Bragadeeswaran, Thangaraj 2011; Esteban 2012; Lazado, Caipang 2014). Primarily secreted in the gut, skin, buccal cavity, nasopharynx, and gills by goblet cells of the epithelium (Hedmon et al. 2018), mucosa also has physiological functions such as aiding in the exchange of nutrients, regulating osmotic and ionic concentrations, minimizing entry of pollutants, and resisting drag when swimming (Esteban 2012; Benhamed et al. 2014; Tiralongo et al. 2020; Ivanova et al. 2022). Its continuous secretion and subsequent shedding, coupled with the activity of exogenous immunocompetent molecules, such as proteases, lysozymes, immunoglobulins, and antibacterial peptides, prevent the stable colonization of pathogens (Lazado, Caipang 2014; Dash et al. 2018; Ivanova et al. 2022). In this accord, numerous studies have been conducted on the antibacterial properties of fish mucus against different pathogens. Skin mucus from the common eel (*Anguilla anguilla*) has antibacterial and hemolytic activities against a wide range of fish pathogens (Bragadeeswaran, Thangaraj 2011). Skin mucus extracts from three cultivable fish species, the Indian carp (*Catla catla*), mrigal (*Cirrhinus mrigala*), and common eel (*Anguilla anguilla*), had antifungal activity against phytopathogenic fungal species, *Aspergillus awamori* and *Colletotrichum falcatum* (Pethkar, Lokhande 2017). Skin mucus extracts of the swamp eel (*Monopterus albus*) were found to be effective against oral and skin pathogens tested on animal models (Hilles et al. 2018; Hilles et al. 2022), as well as fungal pathogens (Ikram, Ridzwan 2013).

The mucosa is a unique transition between the environment and the host, permitting a diverse bacterial community to inhabit it (Larsen et al. 2013; Carda-Dieguez et al. 2017). Some pathogenic strains, like *Aeromonas, Pseudomonas, Streptococcus*, and *Vibrio*, can thrive in the mucosa and cause disease in both wild and reared fish; bacteriosis continues to act as a major bottleneck in natural and artificial aquatic ecosystems (Das et al. 2013; Feng et al. 2017; Nandi et al. 2017; Chen et al. 2018; Doroteo et al. 2018; Xia et al. 2019). The dysbiosis in the mucosal microbiota, especially in the domination of pathogenic groups, predisposes the host to disease (Petersen, Round 2014; Clinton et al. 2021). However, the mucosa can tolerate some commensals that can contribute to orchestrating immune responses against invaders (Gomez et al. 2013; Lowrey et al. 2015; Yu et al. 2021). Some proposed

mechanisms that allow such commensals to fortify defense are through the production of metabolites that kill the pathogens or direct interference with the infection routes to prevent further infection and disease; bacteria-bacteria interaction resulting in population regulation is not a new phenomenon and has been widely explored. There have been studies on fish mucosal antagonists that exhibited activity against different fish pathogens. Some *Pseudoalteromonas* sp. were isolated from the skin mucus of the Indian goat fish (*Parupenus indicus*) and displayed probiotic potential in *in vitro* experiments (Thelma, Asha Devi 2016). *Bacillus cereus* isolated from the skin mucus of calbasu fish (*Labeo calbasu*) was administered with a previously isolated autochthonous intestinal *Aneurinibacillus aneurinilyticus* that had antagonistic activity against *Aeromonas hydrophila* (Bhatnagar, Rathi 2019; Bhatnagar, Dhillon 2023). The synergism resulted in improved growth, immunity, and survival of the host fish. An *Acinetobacter* strain related to *Acinetobacter pittii* isolated from the skin mucus of bighead catfish (*Clarias macrocephalus*) demonstrated strong inhibition of several pathogenic strains *in vitro* (Bunnoy et al. 2019). Most of these studies targeted the skin and gut mucus, and there exists a dearth of studies on the gill mucus (Lazado, Caipang 2014; Reverter et al. 2017). The gill mucus is hypothesized to harbor antagonists that significantly aid in immunity, necessitated by its direct exposure to the constant ingress of pathogens from the external environment (Ringoe, Holzapfel 2000; Chabrillon et al. 2006; Clinton et al. 2021). Its potential for fish health studies is relatively underexplored.

The Asian swamp eel (*Monopterus albus*) is a freshwater fish widely distributed in Asian countries, including China, Malaysia, Indonesia, and the Philippines. Its unique characteristics include the ability to breathe on land, allowing it to move across habitats, having a singular triangular gill slit ventral to its head, being capable of sex reversion, and can tolerate harsh conditions, such as fluctuations in environmental parameters like salinity and temperature, and exposure to a wide array of soil and water pathogens in various habitats (Damsgaard et al. 2014; Hilles et al. 2018; Liu et al. 2019; Xia et al. 2019). Such resilience has attracted interest for its use in aquaculture in other countries, but it is yet to be extensively utilized in the Philippines (Liu et al. 2019). Further, the documented invasive success of the swamp eel around the world can be attributed to its adaptive characteristics and robust immunity, making it a good model species for screening antagonists in the gill mucus (Stevens et al. 2016).

This study aimed to characterize the antagonistic bacterial species present in the gill mucus of the Asian swamp eel (*M. albus*) and utilized a funneled approach to narrow down the species to those that have high antagonistic activity against ubiquitous aquatic pathogens, *Aeromonas hydrophila* and *Vibrio harveyi*, through the spot-on-lawn and co-incubation assays. Characterization included morphological, enzymatic, biochemical, and molecular phases. This study hopes to contribute to the body of knowledge by demonstrating that the gill mucus can be a rich source of antagonistic bacteria against keystone pathogens and can have potential applications in disease management in aquaculture, such as sources of bioactive compounds or use as probiotics. Moreover, the study investigated a relatively untapped field in fish health – the gill mucus, an interesting microenvironment to study as a site of much pathogen ingress in fish.

Materials and methods

Gill mucus collection and bacterial isolation

Six Asian swamp eel (*Monopterus albus* Zuiew) specimens were sourced from a local fish farm in Zarraga, Iloilo, Philippines, and placed in sterile plastic bags with rice field water where they were caught and stored for three days to ensure the isolation of putative autochthonous bacteria. The fish were immobilized through immersion in 50 g L^{-1} sodium bicarbonate solution for 10 min (Caipang et al. 2021) before spiking the head (Reverter et al. 2017). Although the fish were sacrificed at the fish farm, all handling procedures were done in accordance with institutional and national guidelines on proper fish handling and welfare. The ventral surface of the head of the fish was disinfected with 70% ethyl alcohol prior to dissection of the gill slit. The gill mucus from each eel specimen was collected using sterile cotton swabs (Stevens et al. 2016; Clinton et al. 2021; Lorgen-Ritchie et al. 2022) and stored in sterile centrifuge tubes containing 1 mL of normal saline solution. Within 3 h from collection, the gill mucus was subjected to serial dilution and plated on Nutrient Agar (NA) to obtain colonies. After incubation at 28 °C for 22 h, plate counts were performed for each eel specimen, and bacterial isolates with distinct colonial morphology from all plates were pooled and restreaked on fresh agar plates. Subcultures were prepared every two weeks. Eel specimens were sent to the University of the Philippines Visayas Museum of Natural Sciences for confirmation of the target species.

Evaluation of in vitro *antagonistic activity: indicator pathogenic strains*

The isolates were narrowed down to those with *in vitro* antagonistic activity against ubiquitous aquatic pathogens, *Aeromonas hydrophila* and *Vibrio harveyi* (de la Peña et al. 2001), sourced from the Microbiology Laboratory of the University of the Philippines Visayas and Fish Health Section of the Southeast Asian Fisheries Development Center, respectively.

Evaluation of in vitro *antagonistic activity: spot-on-lawn assay*

Nutrient Broth (NB) cultures of the indicator strains were standardized using MacFarland 0.5 turbidity to

approximate 1.5 × 10^8 colony forming units (CFU) per milliliter density (Ikram, Ridzwan 2013; Hilles et al. 2022) for the spot-on-lawn assay. Culture media for *V. harveyi* were accordingly supplemented with 1.5% NaCl to cater to its salt requirements (Doroteo et al. 2018). The standardized culture was inoculated on fresh NA using the spread plate method and allowed to be absorbed by the medium for 1 h. The isolates were then individually spotted on the pathogen lawn and incubated overnight at 28 °C (Caipang et al. 2010). *In vitro* antagonistic activity of an isolate was recorded as a zone of inhibition around it or an abundance of growth over the pathogen lawn. The inhibition zone indicates the exclusion mechanism of antagonism of an isolate where it produces metabolites to exclude the growth of another species within its proximity (Lazado, Caipang 2014). Abundant growth, on the other hand, is representative of the displacement antagonistic mechanism where the isolate is able to displace another species, in this case, the initially inoculated pathogen lawn. Isolates that demonstrated antagonism against both pathogens were subjected to co-incubation assay.

Evaluation of in vitro *antagonistic activity: co-incubation assay*

For the co-incubation assay, the isolates and the indicator strains were plated on NA and subsequently inoculated in NB. Overnight cultures were standardized using UV spectrophotometry (600 nm) and plate count methods and diluted to 10^3 CFU mL^{-1} concentration with normal saline (Doroteo et al. 2018). In a sterile 1.5-mL centrifuge tube, equal aliquots of an isolate and pathogen were added and mixed. For the control, equal amounts of the pathogen and sterile culture media were combined in the tube (Caipang et al. 2023). All tubes were incubated in a rotary incubator at 28 °C, 100 rpm for 24 h.

After incubation, serial dilutions of each tube were prepared and plated on selective culture media: glutamate-starch-phenol red agar supplemented with 20 mg L^{-1} ampicillin (Perales 2003) for tubes co-incubated with *A. hydrophila* and thiosulfate-citrate-bile salts-sucrose agar for *V. harveyi* (Doroteo et al. 2018), for the enumeration of the pathogens following co-incubation (Caipang et al. 2023). The bacterial isolates tested did not grow in both selective media, as determined by preliminary assay; thus, only the pathogens could be counted on the media post-incubation. The plates were incubated at 28 °C for 36 h (Speare, Septer 2019). Plate counts were reported in $\log_{10}$ CFU mL^{-1}. Reduction was indicated by a decrease of $\log_{10}$ CFU mL^{-1} in the count of the pathogen (Caipang et al. 2023). Reduction in pathogen counts in the co-incubated groups was also expressed as a percentage reduction relative to the count in the control setup. The experiments were performed in triplicate. Isolates with remarkable activity were subjected to characterization.

Characterization of bacterial isolates

The bacterial isolates with noticeable antagonistic activity from the co-incubation assay were subjected to morphological, enzymatic, biochemical, and molecular characterization. The first three phases, morphological, enzymatic, and biochemical tests, were performed following the published protocols by the American Society for Microbiology (Smith, Hussey 2005; Breakwell et al. 2007; MacWilliams 2009a; MacWilliams 2009b; McDevitt 2009; Shields, Cathcart 2011; dela Cruz, Torres 2012; Lal, Cheeptham 2012; Reiner 2012).

Macroscopic and microscopic features of the isolates were described. For the macroscopic description, the colony morphology, including colour, form, elevation, margin, opacity, and texture were noted. For the microscopic description, the isolates were Gram-stained, and cell shapes were observed under the microscope at 400× and 1000× magnification. For motility, the isolates were stab-inoculated into a sulfide-indole-motility medium and observed for growth from the stab for one week.

The isolates were tested for the production of enzymes, catalase (3% H_2O_2), amylase (2% starch agar), protease (1% skim milk agar), lipase (1% olive oil agar), gelatinase (12% nutrient gelatin), and urease (urea broth). Culture media formulations were prepared based on the procedures of Simora et al. (2015) and Doroteo et al. (2018).

The isolates were subjected to indole (sulfide-indole medium), methyl red, Vogues-Proskauer tests (methyl red-Vogues-Proskauer broth), citrate (Simmons citrate agar slant), hydrogen sulfide production (sulfide-indole medium), and fermentation of glucose and lactose (triple-sugar-iron agar slant) tests. Isolates were inoculated and observed for characteristic results based on previously described protocols of the American Society for Microbiology.

Molecular identification

For molecular characterization, genomic DNA (gDNA) was extracted from overnight cultures of the isolates in 5 mL broth using a commercial kit (Purelink Genomic DNA Mini, Thermo Fisher Scientific, California, USA) following the instructions of the manufacturer. Extracted gDNA was analyzed using NanoDrop spectrophotometry to ensure sample quality. 16S DNA was amplified using universal primers (Forward: GAGAGTTTGATCCTGGCTCAG; Reverse: CTACGGCTACCTTGTTACGA) (Bianciotto et al. 2003). The 25 µL PCR was comprised of 2 µL (10 to 15 ng) DNA as template, 2 µL of each primer (5 pmol), 2.5 µL PCR buffer, 1.5 µL 2 mM dNTP, 1 µL 50 mM $MgCl_2$ and distilled water. PCR amplification was performed following the protocol of Caipang et al. (2010), and the products were sent for sequencing (Macrogen, Korea). Each isolate was putatively identified using BLASTn search. 16S rRNA sequences of related strains were downloaded from NCBI Genbank (blast.ncbi.nlm.nih.gov) and aligned using the

Table 1. Number and percentage of the bacterial isolates that displayed *in vitro* antagonistic activity against *Aeromonas hydrophila* and *Vibrio harveyi* evaluated using spot-on-lawn assay. Data are shown as number of bacterial colonies and their corresponding percentages out of the 500 bacterial colonies. Exclusion characteristic is qualified when there is a zone of inhibition around the bacterial isolate over the pathogen lawn. Displacement characteristic was indicated by abundant growth of the bacterial isolate on the pathogen lawn

Pathogen	*In vitro* antagonistic activity against the pathogen	
	Number of bacterial colonies exhibiting exclusion characteristic	Number of bacterial colonies exhibiting displacement characteristic
Aeromonas hydrophila	11 (2.2%)	129 (25.8%)
Vibrio harveyi	14 (2.8%)	139 (27.8%)
Both pathogens	64 (12.8%)	

ClustalW method of MEGA 11.0 software (Tamura et al. 2021). A maximum likelihood phylogenetic tree with 1000 bootstrap replications was constructed using IQTREE (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulus et al. 2016) and visualized using iTOL software (https://itol.embl.de/) (Letunic, Bork 2021).

Data analysis

In a co-incubation assay, colony counts (expressed as $\log_{10}$ CFU mL^{-1}) were analyzed using one-way ANOVA (Systat version 8; Systat Software Inc., San Francisco, CA, USA) to compare co-incubated and control treatments. Where ANOVA indicated significant differences, Student's *t*-tests were performed for pairwise comparisons. A significance level of $p \leq 0.05$ was applied for all statistical analyses.

Results

Bacterial isolates in the gill mucuss

The gill mucus of the Asian swamp eel had a bacterial count ranging from 10^4 to 10^6 CFU mL^{-1} as derived from the plate count of the six specimens. A total of 500 bacterial isolates with distinct morphology were subcultured from the dilutions of the gill mucus from all specimens.

In vitro *antagonistic activity of the isolates*

Five hundred isolates were subjected to parallel spot-on-lawn assays against the indicator (pathogenic) strains. A total of 11 isolates had a zone of inhibition against *A. hydrophila*, while 14 isolates presented a zone of inhibition against *V. harveyi* (Table 1). In this assay, the presence of a zone of inhibition suggests an antagonistic mechanism of exclusion. Conversely, displacement is characterized by prolific growth of the isolate over the pathogen lawn. A total of 64 bacterial isolates (12.8%) exhibited some form of antagonistic activity, either exclusion or displacement, against the two bacterial pathogens.

Of the 64 isolates that had activity against both pathogens, eight isolates were tested using co-incubation assay to quantitatively evaluate their antagonistic activity against the two pathogens. The narrowing down in the number of isolates was based on the preliminary assay of the growth of the isolates on the selective media that were used to check for the growth of the indicator pathogens. Following co-incubation with various bacterial isolates, a statistically significant reduction was observed in the population density of both *A. hydrophila* and *V. harveyi*. Specifically, the *A. hydrophila* counts decreased by 6.6 to 22.5%, while *V. harveyi* counts were reduced by 18.3 to 29.7%. Five isolates with the highest mean reduction percentages were subjected to subsequent characterization methods. These chosen isolates reduced the pathogen count by more than 20%.

Table 2. *Aeromonas hydrophila* and *Vibrio harveyi* bacterial counts and their reduction (mean ± SD) after co-incubation with different bacterial isolates. Pathogen counts after co-incubation are reported in log10 CFU mL^{-1}. Reduction in pathogen count was also reported in percentages relative to the control. * indicates a significant reduction in bacterial count at $p < 0.05$

Isolate	*A. hydrophila*		*V. harveyi*		Mean reduction in pathogen count
	Counts	Reduction (%)	Counts	Reduction (%)	
Control	8.60 ± 0.14	–	8.58 ± 0.20	–	–
A13	6.79 ± 0.20*	21.00 ± 0.20	6.88 ± 0.09*	19.80 ± 0.09	20.4
C4	6.67 ± 0.32*	22.50 ± 0.30	6.48 ± 0.01*	24.40 ± 0.01	23.4
C16	7.02 ± 0.14*	18.40 ± 0.14	7.00 ± 0.24*	18.30 ± 0.24	18.4
C24	6.90 ± 0.25*	19.80 ± 0.25	6.71 ± 0.10*	21.80 ± 0.10	20.8
C25	7.29 ± 0.16*	15.20 ± 0.16	6.39 ± 0.56*	25.50 ± 0.56	20.4
C27	7.13 ± 0.02*	17.20 ± 0.02	6.56 ± 0.12*	23.50 ± 0.12	20.3
C30	8.03 ± 0.05*	6.60 ± 0.05	6.52 ± 0.25*	24.00 ± 0.25	15.3
C64	6.91 ± 0.14*	19.70 ± 0.14	6.03 ± 0.05*	29.70 ± 0.05	24.7

Table 3. Morphological, enzymatic, and biochemical characterization of the selected bacterial isolates from the gill mucus

Characteristic		Isolate				
		A13	C4	C24	C25	C64
Morphological	Gram stain	–	–	–	–	–
	Cell shape	rod	rod	rod	rod	rod
	Color	yellowish	white	yellowish	yellowish	white
	Form	irregular	circular	circular	circular	circular
	Elevation	raised	flat	flat	flat	flat
	Margin	wavy	entire	entire	entire	entire
	Opacity	translucent	translucent	translucent	translucent	translucent
	Texture	matte	moist	moist	moist	moist
	Motility	+	–	–	–	–
Enzymatic	Catalase	+	+	+	+	+
	Amylase	+	–	+	+	–
	Protease	+	+	–	–	–
	Lipase	+	+	+	+	+
	Gelatinase	+	+	–	–	+
	Urease	–	–	–	–	–
Biochemical	Indole test	+	–	–	–	–
	Methyl red	+	–	+	+	–
	Vogues-Proskauer	+	–	–	–	–
	Citrate test	+	+	+	+	+
	H_2S production	–	–	–	–	–
	Glucose fermentation	+	–	–	–	–
	Lactose fermentation	+	–	–	–	–

Characterization of the isolates

The results of the characterization assays are reflected in Table 3. All five isolates were Gram-negative and bacillus-shaped. A13 had a distinct colony morphology compared to the rest having a yellowish colony colour, irregular form, raised elevation, wavy margin, translucent opacity, and matte texture; C4, C24, C25, and C64 had similar colonial characteristics of white or yellowish colony colour, circular form, flat elevation, entire margin, translucent opacity, and moist texture. The isolates were able to produce at least three of the six extracellular enzymes tested. All isolates were positive for catalase, lipase, and citrate tests. Isolates A13, C4, and C64 produced gelatinase. None produced hydrogen sulfide and urease. Only A13 was positive for all enzymatic tests except for urease and was the only isolate that fermented glucose and lactose.

Molecular characterization of the isolates revealed that A13 was a putative *Aeromonas*, while the rest were *Pseudomonas*. From the BLASTn alignment (Table 4) and phylogenetic analysis (Fig. 1), the identities of the isolates were inferred: A13 was 93% identical to the sequence of the strain 202108B3 of *Aeromonas dhakensis*, while C4 was 95% similar to the sequence of strain D116_SP6R of *Pseudomonas azotoformans* supported by a clear sub-group in the phylogenetic tree. Both C24 and C25 were highly similar to putative *Pseudomonas parafulva* (strain PRS09-11288) with percentages, 98% and 96%, respectively. Lastly, C64 was 97% identical to strain NBSII of *Pseudomonas gessardii*.

Discussion

The fish mucosa provides protection to the host through the activity of immunocompetent molecules and metabolites, as well as the antagonism of commensal bacteria against pathogens (Lazado, Caipang 2014; Das et al. 2018; Ivanova

Table 4. Molecular identification of the bacterial isolates from the gill mucus based on BLASTn search (16S rRNA)

Isolate	Sequence length (bp)	Highest identity	Strain code	GenBank accession No.	Identities (match/total)	Percentage similarity	Query cover
A13	1100	*Aeromonas dhakensis*	202108B3	OQ283677.1	1041/1125	93%	99%
C4	1185	*Pseudomonas azotoformans*	D116_SP6R	MK883209.1	1036/1095	95%	92%
C24	1185	*Pseudomonas parafulva*	PRS09-11288	CP019952.1	1011/1030	98%	86%
C25	1206	*Pseudomonas parafulva*	PRS09-11288	CP019952.1	1098/1142	96%	94%
C64	1198	*Pseudomonas gessardii*	NBSII	KT184489.1	1117/1151	97%	95%

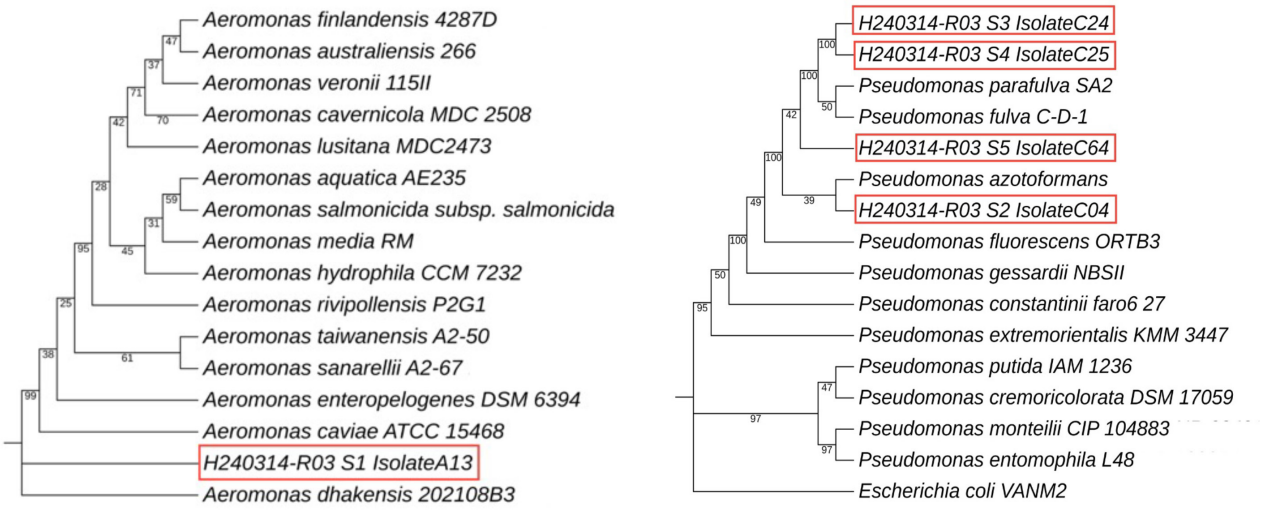

Fig. 1. Phylogenetic trees of the *Aeromonas* and *Pseudomonas* isolates from the gill mucus of the swamp eel. Trees were generated based on maximum likelihood inference with 1000 bootstrap replications. Sequence data of the isolates were aligned with related strains from BLASTn search in NCBI.

et al. 2022). The findings of this research support the hypothesis that the gill mucus can be a good source of antagonistic bacteria aside from the gut and skin mucosa. From this study, around 12.8% of the isolates screened exhibited antagonism against the tested aquatic pathogens, *A. hydrophila* and *V. harveyi* in the spot-on-lawn assay, comparative to the results obtained by previous studies that screened autochthonous antagonists (Caipang et al. 2010; Stevens et al. 2016; Caipang et al. 2022; Bhatnagar, Rathi 2023). To quantitatively evaluate the antagonistic activity, the five isolates that were subjected to co-incubation assay for further characterization reduced pathogen count by at least 20%, similar to the percentages obtained by Caipang et al. (2023) on the isolation of potential probionts with anti-*V. harveyi* activity. Antagonism against the indicator pathogens can be due to mechanisms such as competition, displacement, or exclusion (El-Saadony et al. 2021). Competition can be one of the plausible mechanisms for the antagonism observed since the pathogen and the antagonistic bacteria were co-incubated in equal amounts (Lazado et al. 2011), apart from exclusion and displacement mechanisms displayed by the isolates in the spot-on-lawn assay.

From this study, the five isolates that had notable activity from the antagonism assays performed were characterized by their morphological, enzymatic, biochemical, and molecular profiles, which revealed that they were putative *Aeromonas* and *Pseudomonas*. Antagonism is an important prerequisite property of probiotics, and these genera are among the common sources of probiotics that have the potential for use in aquaculture, along with *Lactobacillus, Leuconostoc, Enterococcus, Carnobacterium, Shewanella*, and several others (Ringoe, Holzapfel 2000; Kesarcodi-Watson et al. 2007; Nayak 2010; Allameh et al. 2012; Teneva et al. 2016; Yu et al. 2021). *Pseudomonas* encompasses ubiquitous strains found in soil and aquatic environments and even animal tissues (Burr et al. 2010; Lauritsen et al. 2021). It is an integral member of the core microbiota of different tissues and mucosal layers of several fish species (Larsen et al. 2013; Leonard et al. 2014; Kearns et al. 2017; Reverter et al. 2017; Rosado et al. 2018; Chen et al. 2019; Rosado et al. 2023), having purported contributions to the physiological functions of the host, including nutrition, metabolism, host-microbe interactions, and immunity (Nayak 2010; Stevens et al. 2016; Chen et al. 2019). Although *Pseudomonas* harbours several strains that are pathogenic, previous studies have isolated strains with their secreted secondary metabolites that presented biocontrol and probiotic properties (Heikkinen et al. 2014; Mancuso et al. 2015; Rizzo et al. 2020; Lauritsen et al. 2021; Zheng et al. 2021; Oni et al. 2022).

The isolated strains are putative *P. azotoformans, P. parafulva*, and *P. gessardii* and have previously been reported to have activity against a wide range of bacterial and fungal pathogens in rice and cucumber (Wulff et al. 2010; Sang et al. 2014; Liu et al. 2015; Fang et al. 2016; Hofte 2021; Zheng et al. 2021). The concurrent isolation of these strains in rice and the swamp eel is thought to be a result of a shared ecological niche in a rice paddy habitat. All isolates were able to produce at least one beneficial enzyme among catalase, amylase, protease, and lipase, which are beneficial for eventual probiotic application (El Saadony et al. 2021; Rodrigues et al. 2021; Caipang et al. 2022; Khushboo et al. 2023). Further, all isolates can utilize citrate as a sole energy source. Isolates C4 and C64, putative *P. azotoformans* and *P. gessardii*, respectively, produced gelatinase, which is highly associated with pathogenicity and virulence in bacteria (Rodrigues et al. 2021).

Interestingly, one of the isolates (A13) characterized had high sequence similarity with *Aeromonas dhakensis*, an

emerging fish and human pathogen. Of all the isolates, this had the lowest sequence similarity possibly consequential to the misidentification of *A. dhakensis* as other *Aeromonas* strains, such as *A. hydrophila, A. caviae*, and *A. veronii* in previous isolation studies and discrepancies in biochemical profiling (Martinez-Murcia et al. 2008; Aravena-Roman et al. 2011; Esteve et al. 2012; Beaz-Hidalgo et al. 2013; Chen et al. 2016; Teunis, Figueras 2016). Using genomic data, it has no unambiguous signature to distinguish it from *A. caviae*, as seen with the outgroup cluster in the phylogenetic tree (Chen et al. 2016). It is also possible that the isolated strain in this study is a novel species, but this can only be confirmed by further characterization to define its biological properties. *Aeromonas* is a repository of opportunistic pathogenic strains widely distributed in different fish species (Chen et al. 2016; Xia et al. 2019; Rathinam et al. 2022). Particularly, *A. dhakensis* has been isolated as a predominant species in diseased wild and farmed eels (Esteve et al. 2012; Yi et al. 2013). In this study, the isolated strain tested positive for motility, catalase, amylase, protease, lipase, gelatinase, and carbohydrate fermentation, providing a survival advantage in various habitats (Fernández-Bravo, Figueras 2020; Khushboo et al. 2023). Converging evidence over the past years proves that *A. dhakensis* infections exhibit greater virulence than other *Aeromonas* infections (Chen et al. 2016). However, this is the first documentation of the antagonism of *A. dhakensis* against other pathogens. Another bacterium, *Pseudomonas aeruginosa*, is a serious pathogen in humans, but has a strain that has reported antagonism against pathogens in economically important crops (Shi et al. 2015). This might be the same case with the isolated *A. dhakensis* and can be potentially harnessed for potent bioactive compounds.

However, it should also be noted that the observed antagonism of a bacterial species should not mask the possibility that it can still be pathogenic, eliciting the importance of *in vivo* studies involving pathogenicity and survival experiments. Corollary to this finding is that some species have selective antagonism against different pathogens, wherein they promote the growth of other pathogens while reducing the population of another (Xu et al. 2023). It is also possible that such bacterial groups can convert into pathogenic groups due to alterations in the host microbiota structure (Gan et al. 2021). Antagonistic effects and disease occurrence in aquaculture are not only implicated due to the virulence of the pathogen but also encompass multifactorial interactions with other species in the microbiota of the host. In-depth analyses of such interactions are necessary to magnify desired antagonistic effects against pathogens and mitigate counteractive events.

Conclusions

Taken together, using Asian swamp eel as a model, the gill mucus, an underexplored mucosal surface, is a rich source of bacterial isolates with antagonistic properties that have great potential applications for biocontrol in aquaculture. As the first study to screen antagonists in the gill mucus of the swamp eel, and one of the few studies that studied the gill mucus of fish species, in general, this encourages more investigations to be conducted on the gill mucus and its implications in fish health. From this study, the isolates that can be subjected to further characterization would be C24 and C25, which are putative *P. parafulva*. For the other isolates, their presence in the swamp eel and documented antagonistic activity may be leveraged for further investigation of their contributions as antagonists against pathogens. A future direction for this study is the probiotic characterization of the isolates, highlighting *in vivo* studies in order to elucidate their potential effects on the growth, metabolism, and survival of fish.

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