

Genomic Characterization of Virulence and Resistance Genes in Bacterial Isolates from Lettuce Irrigation Water

Nailus Syarifah¹, Siti Nurjanah^{1,2*}, Harsi D. Kusumaningrum^{1,2} and Winiati P. Rahayu^{1,2}

¹Division of Food Science and Technology, Faculty of Engineering and Technology, IPB University, Darmaga Campus, Bogor 16680, Indonesia

²South-East Asia Food and Agricultural Science and Technology (SEAFAST) Center, IPB University, Darmaga Campus, Bogor 16680, Indonesia

Abstract. Antibiotic resistance and virulence genes in the environment pose significant risk to food safety, particularly in fresh produce that is commonly consumed raw, such as lettuce. Lettuce agriculture is highly dependent on irrigation water, which can serve as a crucial reservoir for bacteria that transmit antibiotic resistance and virulence factors. Tetracycline is among the most commonly detected antibiotics in agriculture. This has an impact on the presence of bacteria that carry resistance and virulence genes in water systems. This study aimed to characterize the genomic profile, including virulence, antibiotic resistance genes, and plasmids of bacteria isolated from lettuce irrigation water. This study consists of two parts: part 1 tests the resistance of 19 bacterial isolates to tetracycline using the Kirby-Bauer method and part 2 confirms the bacterial isolates using real-time PCR. The second step is characterize the genome using Whole-genome sequencing (WGS) using the GenoLab M platform (GeneMind), followed by bioinformatics analysis. Among the 19 isolates tested, 4 (21.05%) were resistant, 1 (5.26%) exhibited intermediate resistance, and 14 (73.68%) were susceptible to tetracycline. All isolates tested negative for the *invA* gene, indicating the absence of *Salmonella* enteric pathogenic strains. WGS-based phylogeny identified H.1.2 as *Pseudomonas peradeniyensis*, harboring 45 virulence-associated and 4 stress-related genes. Resistance profiling revealed the *mexE* (abriTAMR) and *yajC* (CARD) genes. However, no plasmids were detected in H.1.2 isolate based on PlasmidFinder. This indicates that the isolate H.1.2 in this study is not hazardous to humans.

1 Introduction

The global consumption of fresh vegetables, especially lettuce, has been steadily increasing over the past decade. According to Precision Business Insights (2024) [1], the production of lettuce and chicory reached 28 million tons in 2023, with an average per capita consumption of approximately 3.5 kg/person/year. This growth in demand indicates rising consumer awareness and preference for healthy dietary patterns. However, consuming raw or minimally processed lettuce without sufficient hygiene and sanitation practices can pose a significant risk of exposure to microorganisms. Between 2017 and 2019, at least 85 outbreaks associated with fresh fruits and vegetables were reported in the United States, resulting in more than 4,500 cases of illness and more than 1,100 hospitalizations [2]. These findings highlight the critical significance to identify and monitor potential sources of contamination throughout the production and distribution chain. Among these, irrigation water has been identified as a significant environmental reservoir that may facilitate the transmission of microorganisms to fresh vegetables.

Irrigation water is a major reservoir for various microorganisms, including *Salmonella* spp., *Klebsiella* spp., *Escherichia coli*, and *Pseudomonas* spp. *Pseudomonas* sp. is a type of environmental bacteria widely recognized for its strong tolerance to a variety of environmental conditions, including antibiotic exposure [3]. Most *Pseudomonas* species are not enteric pathogens, but their presence in water is important to note as they can contribute to the spread of antibiotic resistance in the environment [4]. Among the antibiotics commonly applied in both crop cultivation and livestock production, tetracycline is widely used to prevent and control bacterial infections [5]. However, excessive or inappropriate exposure to tetracycline may exert selective pressure that promotes the emergence of resistant bacteria and the persistence of antibiotic resistance gene (ARGs) in the environment.

Recent advances in genomic technologies have facilitated the comprehensive detection and characterization of microorganisms and their associated virulence and resistance profiles. Whole-genome sequencing (WGS), in particular has emerged as an effective approach for accurately identifying species and understanding genetic drivers of virulence and antibiotic resistance. The research by Batrich et al. (2019) utilized WGS in identifying microbes in Michigan's freshwater, with *Pseudomonas* being the most commonly found

*Corresponding author: sity_nr@apps.ipb.ac.id

genus. In addition, the presence of virulence and antibiotic resistance genes was also studied [6].

Based on this background, the present study was designed to comprehensively assess tetracycline resistance among bacterial isolates using the disk diffusion (Kirby-Bauer) method, verify the presence of the *invA* gene using real-time PCR, and characterize microbial composition and phylogenetic relationships based on whole-genome sequencing (WGS) data. Furthermore, this study uses bioinformatics analysis to determine the distribution of virulence and antibiotic resistance genes. The findings of this study are expected to provide scientific insights and references for researchers in identifying bacterial species in agricultural water, providing insights into virulence genes and antibiotic resistance genes in bacteria obtained through a genomic approach.

2 Materials and Methods

The study was conducted from January to August 2025 at the Food Microbiology II laboratory and the Molecular Microbiology Laboratory, PAU, Faculty of Agricultural Engineering and Technology, IPB University. A total of 19 bacterial isolates collections were previously obtained from irrigation water in conventional and hydroponic lettuce cultivation systems in Bogor. Tetracycline susceptibility testing was performed using the Kirby-Bauer disk diffusion method to evaluate the sensitivity of isolates to commonly applied environmental antibiotic. The disks used contained 30 µg of tetracycline antibiotic (Oxoid). Antibiotic susceptibility was tested by measuring the diameter of the inhibition zone generated around the tetracycline (30µg) disc. According to the Clinical and Laboratory Standards Institute guidelines, isolates were categorized as resistant (≤ 11 mm), intermediate (12-14 mm), or sensitive (≥ 15 mm) [7]. These classifications provide information on each isolate's response to antibiotic exposure. In parallel, real-time PCR (CFX Opus 96) targeting the *invA* gene was performed to confirm bacterial identification of enteric pathogen *Salmonella* bacteria. The *invA* gene is a marker of the *Salmonella* genus, as it encodes a component of secretion system III that plays a role in bacterial invasion of host cells, and can distinguish *Salmonella* from other bacteria [8].

Genomic DNA was extracted using the ZymoBIOMICS DNA Miniprep Kit (50 prep), and DNA concentration was quantified using a Nanospectrophotometer (Nilab) and Qubit™ 4 fluorometer (Invitrogen). The purity of the extracted DNA was assessed based on $A_{260/280}$ and $A_{260/230}$ absorbance ratios. Whole-genome sequencing (WGS) was performed using the GenoLab M platform (GeneMind Biosciences, China), and further analysis was performed using several bioinformatics tools, including Galaxy (<https://usegalaxy.eu/>), Proksee (<https://proksee.ca/>), CARD (<https://card.mcmaster.ca/analyze/rgi>), RAST (rast.nmpdr.org), and TYGS (tygs.dsmz.de).

3 Results and Discussion

3.1 Tetracycline Resistance in Bacterial Isolates

Detection of antibiotic resistance in bacteria is necessary to determine the level of bacterial sensitivity to antibiotics in the environment. Antibiotic test results can be obtained phenotypically by measuring the inhibition zone formed around the antibiotic disk. The average inhibition zone diameters obtained from the tetracycline susceptibility assay are summarized in Table 1.

Table 1. Mean inhibition zone diameters of bacterial isolates tested against tetracycline

Isolate	Mean Inhibition Zone (mm) \pm SD	Sensitivity Status	<i>tetA</i> *
K.1.1	27.83 \pm 0.51	S	+
K.1.2	23.76 \pm 0.80	S	-
K.3.1	26.94 \pm 0.68	S	-
K.4.1	8.59 \pm 1.23	R	-
K.5.1	10.10 \pm 1.84	R	-
K.5.2	23.30 \pm 1.76	S	-
H.1.2	23.02 \pm 0.76	S	-
H.3.1	25.32 \pm 0.85	S	+
H.3.2	26.67 \pm 1.31	S	-
H.4.1	12.31 \pm 2.46	I	-
H.4.2	23.61 \pm 1.56	S	-
H.5.2	24.84 \pm 0.98	S	-
H.6.1	25.72 \pm 1.44	S	-
H.6.2	11.20 \pm 1.24	R	-
H.7.1	25.48 \pm 1.31	S	+
H.8.1	24.62 \pm 1.38	S	-
H.9.1	24.14 \pm 1.09	S	-
H.9.2	24.48 \pm 1.08	S	-
H.10.1	8.92 \pm 0.65	R	-

Note: S: Sensitive, I: Intermediate, R: Resistant

*The study by Permatasari (2025) reported the detection of the *tetA* gene in lettuce irrigation water using conventional PCR [9].

Currently, the use of tetracycline antibiotics in agriculture is still widespread, causing contamination of irrigation water. As shown in Table 1, among the 19 bacterial isolates analyzed, 4 isolates (21.05%) exhibited resistance to tetracycline, 14 isolates (73.68%) were sensitive, and 1 isolate (5.26%) showed an intermediate response. Resistance to tetracycline is mainly mediated by specific resistance genes such as *tetA*, *tetB*, *tetC*, and *tetE*, which encode efflux pump systems, or *tetM*, which encodes ribosomal protection proteins. These mechanisms allow bacteria to actively expel antibiotics from cells or inhibit the binding of antibiotics to ribosomal targets [10].

The percentage of resistant isolates observed in this study is consistent with the findings of Mukherjee *et al.* (2020), who reported a higher prevalence of antimicrobial resistance in water compared to soil, with 27% of bacterial isolates showing resistance to

tetracycline [11]. The presence of an intermediate isolate in the current investigation may also indicate a risk of full resistance developing after prolonged or repeated antibiotic exposure. These findings highlight the importance of regular monitoring of resistance dynamics in agricultural water systems in order to reduce the environmental spread of antimicrobial resistance.

Differences were found between the antibiotic susceptibility test results obtained from the disk diffusion method (phenotypic test) and the results obtained from conventional PCR (genotypic test) in isolates K.1.1, K.4.1, K.5.1, H.3.1, H.6.2, H.7.1, and H.10.1. Specifically, isolates K.1.1, H.3.1, and H.7.1 showed a sensitive phenotype in the disk diffusion test, but were identified as resistant in the genotypic test (Table 1)[9]. This discrepancy may be due to the presence of the *tetA* gene that is not expressed under test conditions, or a mutation that renders the gene inactive, resulting in the absence or malfunction of the efflux pump protein [12]. Consequently, even though the isolates harbor the *tetA* gene, they may still exhibit phenotypic susceptibility to tetracycline in the disk diffusion test.

In contrast, isolate K.4.1, K.5.1, and H.6.2 showed phenotypic resistance but negative results for the *tetA* gene the PCR assay (Table 1) [9]. This can be explained by the presence of alternative *tet* genes, such as *tetB*, *tetC*, *tetD*, *tetM*, *tetX*, or others, which were not targeted in the PCR analysis. When the PCR assay is limited to detecting only the *tetA* gene, other *tet* variants remain undetected, resulting in negative genotypic results despite a resistant phenotype. The absence of detectable resistance genes in phenotypically resistant isolates may also be due to uncertain phenotypic resistance, low gene expression levels, or the involvement of other non-genetic resistance mechanisms [13].

3.2 The Presence of the *InvA* Gene in Bacterial Isolates

Genotypic identification of isolates was performed using real-time PCR targeting the *invA* gene.

Table 2. Detection of the *invA* gene in bacterial isolates by real-time PCR

Isolate	C _T value	Isolate	C _T value
K.1.1	N/A	H.4.2	34.34
K.1.2	32.78	H.5.2	N/A
K.3.1	33.05	H.6.1	N/A
K.4.1	N/A	H.6.2	N/A
K.5.1	N/A	H.7.1	34.39
K.5.2	32.48	H.8.1	31.08
H.1.2	31.07	H.9.1	N/A
H.3.1	32.33	H.9.2	32.38
H.3.2	33.04	H.10.1	N/A
H.4.1	N/A		

Real-time PCR results were expressed as cycle threshold (C_T) values, which indicate the number of amplification cycles required for the fluorescence signal

to exceed the detection threshold. Lower C_T value indicate higher concentration of target DNA [14].

Table 2 shows the detection results of the *invA* gene using real-time PCR. According to Thilakarathna *et al.* (2022), the C_T values above the cut-off threshold. A N/A result indicates that the isolates does not contain the *invA* gene (negative), whereas isolates with C_T values higher than 31 suggest the presence of very low DNA concentrations, necessitating further confirmation [15]. Considering these results, isolate H.1.2, which showed a C_T value close to the cut-off (31.07), was selected for further analysis using Next-Generation Sequencing (NGS). The H.8.1 isolate was not selected despite also showing a C_T value close to the threshold (31.08), because the difference between the two was minor (0.01). This difference was not significant and was therefore considered to provide no significant biological information.

3.3 Bacterial Isolates and DNA Sequencing

The selected isolate proceeded to the next stage, which was bacterial extraction and sequencing. The whole-genome sequencing (WGS) result of isolate H.1.2 revealed a total genome length of 5,795,373bp, consisting of 130 contigs, with a GC content of 64.82% and an N50 value of 115,416bp. These characteristics are comparable to the *Pseudomonas putida* reference genome available in the NCBI database, which reports a total genome size of 5.7 Mb, 244 contigs, a GC content of 62%, and an N50 of 169 kb [16], and *Pseudomonas peradeniyensis* had a percentage GC of 64.62 [17]. Genome completeness, evaluated using BUSCO, showed a high completeness score of 99.2%, indicating a nearly complete assembly as it was within the expected threshold of ≥95 [18].

3.4 Visualization and Genome Annotation

In this study, genome visualization was also performed using the web-based PROKSEE platform. The sequence data uploaded to PROKSEE generated several assembly metrics and graphical outputs, including circular genome maps presented in Figure 1. These maps provide an overview of the chromosome structure of each isolate and its alignment with the reference, with related gene components serving as a standard representation of bacterial genomic organization.

As shown in Figure 1, the green circles indicate matches with the NCBI database, while the dark blue circles indicate the identification of genes present in the isolate. The correspondence between the green and blue circles indicates that the isolate genome matches the bacterial genomes already present in NCBI. The innermost layer of the circular genome map represents GC content, which indicates the proportion of guanine (G) and cytosine (C) bases throughout the genome. The subsequent layer corresponds to GC skew, which determines contig boundaries and assists in the interpretation of genomic features [19].

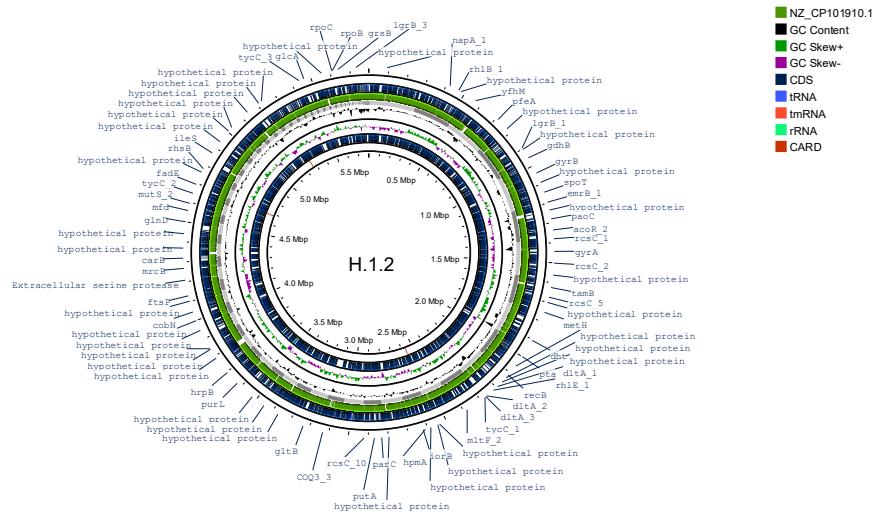


Fig. 1. Genomic circular representation of isolate H.1.2, PROKSEE visualization. NCBI reference genome (dark green), GC content (black), GC skew+ (dark green), GC skew- (purple), CDS (dark blue), tRNA (blue), tmRNA (red), rRNA (light green), CARD (brick red)

The dark blue annotations indicate the predicted gene features obtained from Prokka annotation. RNA elements, including tRNA, rRNA, ant tmRNA, are also represented along with several “hypothetical protein” genes, which refer to predicted open reading frames

(ORFs) with uncharacterized functions. Additionally, several identified coding genes such as *ropB*, *sucA*, and *gyrA* were also detected, representing conserved functional components within the bacterial genomes.

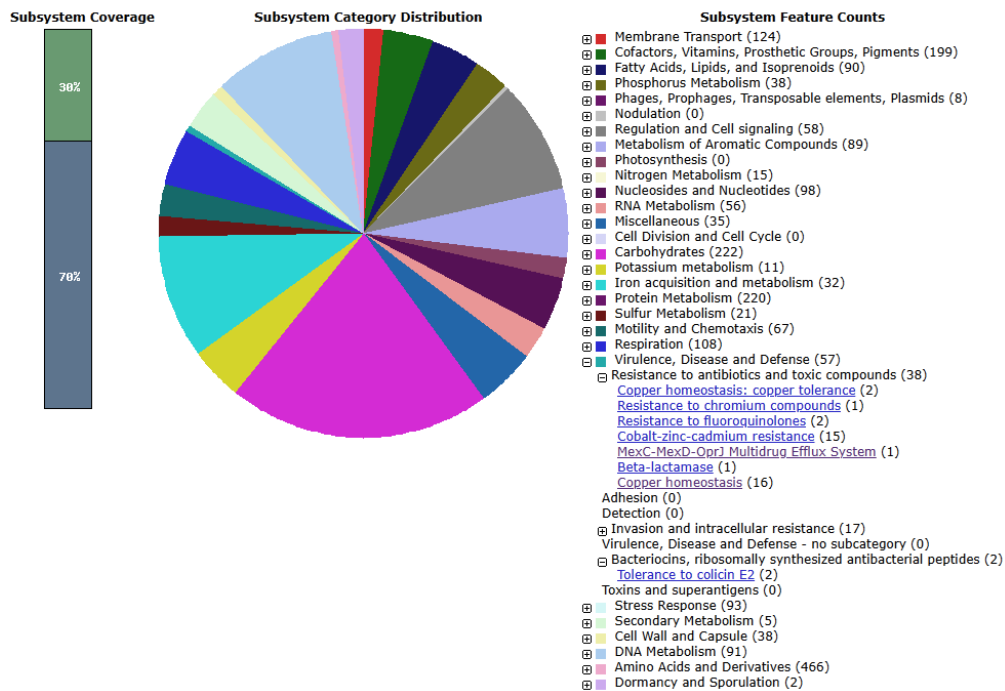


Fig. 2. Subsystem distribution of genes based on RAST annotation

The Rapid Annotation using Subsystem Technology (RAST) server, developed under The SEED Project, is one of the automated platforms designed for microbial genome annotation [20]. Subsystem-based genome annotation provides a functional framework for exploring the biological potential and metabolic capabilities of microorganisms (Figure 2).

As illustrated in Figure 2, the green segments on the left represent features classified within defined subsystems, whereas the blue segments indicate features that are not assigned to any known subsystem [21]. This study focused on the category “Virulence, Disease, and Defense”, which includes genes that contribute to pathogenicity or potential virulence traits.

In this category, isolate H.1.2 exhibits virulence, disease, and defense in 57 of 2,243 genes, or 2.54%.

these genes include invasion and intracellular resistance in 17 genes, resistance to antibiotics and toxic compounds in 38 genes, and details of 1 gene *mexC-mexD-oprJ* multidrug efflux system, 1 chromium component resistance gene, 1 β -lactamase gene, 15 heavy metal resistance genes for cobalt-zinc-cadmium, 16 copper homeostasis genes, 2 copper tolerance genes, and 2 fluoroquinolone genes. RAST categorizes genes into broad functional categories, with the majority focusing on heavy metal tolerance and cellular homeostasis for environmental adaptation rather than direct antibiotic resistance. To confirm RAST results,

additional tools such as abriTAMR, nad AMRFinderPlus can identify virulence and antibiotic resistance genes.

3.5 Species Identification

Taxonomy profiling was performed to determine the species of the bacterial isolate. The phylogenetic tree was constructed with the help of the Type Strain Genome Server (TYGS) website (Figure 3).

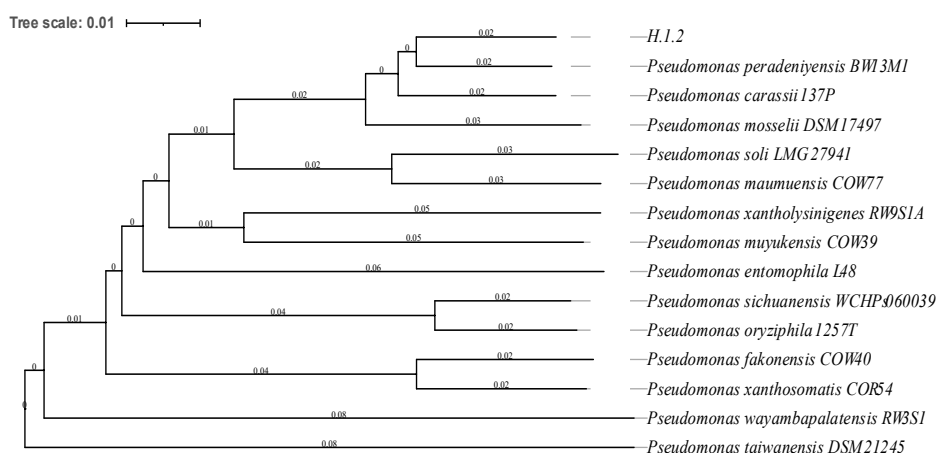


Fig. 3. Phylogenetic Tree Constructed Using the Type Strain Genome Server (TYGS)

Genome-based phylogenetic analysis revealed that isolate H.1.2 clustered closely with *Pseudomonas peradeniyensis* BW13M1, followed by *P. peradeniyensis* has been described as a member of the *Pseudomonas putida* sensu lato group [17]. Girard et al. (2021) reported the identification of numerous new species in the *P. putida* group created using *rpoD* and full genome phylogeny datasets. *P. peradeniyensis* is a new species found among previously defined strains of *P. putida*, according to the findings [17]. This finding indicate that the H.1.2 isolate belongs to or is closely related to the *P. putida* group.

3.6 Identification of Virulence and Antibiotics Resistance Genes

The identification of virulence genes was performed using ABRicate with the VFDB database, while stress-related genes were detected using AMRFinderPlus. Antibiotic resistance genes (AMGs) were analyzed using CARD, abriTAMR, and AMRFinderPlus database. Using ABRicate and the VFDB database, the H.1.2 isolate was found to harbor 45 virulence-associated genes and four stress-related genes (*ttgB*, *ttgA*, *ttR*, and *cadR*). However, none of the detected genes were classified as classical invasion factors, cytotoxins, toxin-producing genes, or host damage

determinants typically associated with direct human pathogenicity. Most of the identified genes were related to intrinsic processes such as environmental adaptation, regulatory proteins, and efflux systems. This suggests that the virulence profile of H.1.2 isolate is more related to environmental adaptation than to direct pathogenicity.

Among these genes, *algU*, *mucD*, *algW*, *algA*, *algC*, *alg8*, *algI*, *algS*, and *algB* genes are grouped into the alginate biosynthesis cluster. This cluster plays a key role in the formation of mucoid biofilms and contributes to increased resistance to antibiotics and phagocytosis [22,23]. The genes such as *pvdS*, *pvdH*, *pvdM*, and *mbtH-like* were identified as components of the iron acquisition system, which is essential for the survival and pathogenicity of bacteria under iron-limiting conditions [24]. In addition, the detection of *pscS* and *pscR* genes indicates the presence of a type III secretion system (T3SS) apparatus, a critical virulence mechanism that mediates the translocation of effector proteins into host cells during acute infection [25]. Conversely, the presence of the *hsiB1/vipA*, *hsiCq/vipB*, *hcp1*, *hsiG1*, *clpV1*, *dotU1*, and *tagR* genes suggests the presence of an active type VI secretion system (T6SS), which contributes to interbacterial competition and facilitates interactions with host cells [26].

The identification of antibiotic resistance genes (ARGs) was performed using the Comprehensive Antibiotic Resistance Database (CARD) (Table 3).

Based on the CARD curated detection models, the predicated resistance genes were categorized into three confidence levels: perfect, strict, and loose hits [27]. Based on the results, the H.1.2 isolate as *Pseudomonas* exhibited two genes detected as strict hits, namely *YajC* with a percent identity (%ID) of 91.07%. In addition, at least 472 genes were classified under the loose hits category. The *YajC* gene is near *secD* and *secF*, which are known to facilitate protein translocation and membrane protein assembly. Together, *secD*, *secF*, and *YajC* from a membrane-associated complex involved in the maturation and export of several exoproteins and membrane proteins that contribute to adhesion and stress response. Therefore, changes in the expression or structural integrity of *YajC* may affect the ability of bacteria to adapt to aquatic environments or host tissues.

In this study, the detection of the *yajC* gene in an isolate obtained from irrigation water suggests that this gene may support bacterial persistence under environmental stress conditions, such nutrient limitation and fluctuating water environments. Additionally, according to Duman *et al.* (2024), *YajC* exhibits a broad spectrum of resistance, including resistance to fluoroquinolones, cephalosporins, glycolcyclines, penams, tetracycline, oxazolidinones, glycopeptides, rifamycins, phenicol antibiotics, as well as disinfectant and antiseptic agents [28]. In general, the H.1.2 isolate in this study has resistance-associated genes with shared adaptation mechanisms, but lacked particular antibiotic resistance.

In addition to CARD, analysis using the abriTAMR and AMRFinderPlus tools detected one resistance gene (Table 3), *mexE*, which is associated with the efflux pump mechanism.

Table 3. Antibiotic resistance gene in H.1.2 isolate

AMR Gene		
abriTAMR	AMRFinderPlus	CARD
<i>mexE</i>	<i>mexE</i>	<i>yajC</i>

The *mexE* gene functions together with *mexF* and *oprN* to efflux various toxic substrates, including several classes of antibiotics, thereby contributing to multidrug resistance. Beyond its role in antimicrobial resistance, the *mex* operon also performs important physiological functions, such as exporting signaling molecules and metabolites that influence virulence, biofilm formation, and cellular homeostasis in *Pseudomonas aeruginosa*. Consequently, changes in *mexE* expression may have broad effects on bacterial phenotypes, affecting both environmental persistence and host interactions [29]. Several studies have reported several genes associated with antibiotic resistance in *Pseudomonas* sp., such as *fosA*, *aph(3')-Iib*, *bla_{OXA-50}*, *catB7* [30], *mexA*, *mexB*, *mexZ* [31], *mexC*, and *mexE* [32].

3.7 Plasmid Identification

Plasmids are among the key mobile genetic elements (MGEs) that facilitate the horizontal transfer of various genes, including genes that confer antibiotic resistance

[33]. The presence of plasmids in H.1.2 isolate was not detected, indicating that the antibiotic resistance and virulence genes identified in this isolate are most likely encoded in the chromosome rather than carried by plasmids. This finding is in line with the research by Tran *et al.* (2022), which stated that no plasmids were found in the *Pseudomonas putida* TT321 and TT322 isolates [34]. The study by Molina *et al.* (2014) indicates that the genes causing antibiotic resistance in *P. putida* HB3267 can be found in two locations, the chromosome and the plasmid [35].

The results of this study indicate that the presence of virulence and resistance genes in isolates is not necessarily dependent on plasmids. Furthermore, the absence of plasmids suggests that the H.1.2 isolate has a poor chance of horizontally transferring virulence and antibiotic resistance genes to other bacteria. This is because the presence of plasmids is frequently connected with pathogenicity and AMR transmission in bacteria capable of infecting humans or clinical environments. As a result, according to this, the H.1.2 isolate in this investigation can be classified as safe for humans.

4 Conclusion

The results of this study demonstrated that among 19 bacterial isolates from lettuce irrigation water, four (21.05%) exhibited resistance to tetracycline, one (5.26%) exhibited intermediate resistance and fourteen (73.68%) were susceptible to tetracycline, as determined by the Kirby-Bauer disc diffusion method. Following confirmation, all of the isolates lacked the *invA* gene, indicating they did not belong to the *Salmonella* enteric pathogen group. Through genomic analysis, isolate H.1.2 was identified as *Pseudomonas peradeniyensis*. ABRicate and AMRFinderPlus found that isolate H.1.2 carries 45 virulence genes and 4 stress genes. Isolate H.1.2 harbored the *mexE* (abriTAMR) and *yajC* (CARD) genes, which linked adaptation pathways, but lacked antibiotic resistance. PlasmidFinder identified no plasmids in isolate H.1.2. This indicates that isolate H.1.2 is more likely to adapt to survive in the environment than to cause direct infection in humans.

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