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Complex resistance mechanisms in multidrug-resistant *Pseudescherichia vulneris* from a Healthy Cat: Unveiling genomic architecture and public health implications

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ABSTRACT

Antimicrobial resistance is a critical global public health challenge. *Pseudescherichia vulneris* is an opportunistic pathogen that carries resistance genes and can invade and colonize various sites in both humans and animals. In this study, we characterized a strain of *P. vulneris* isolated from the intestinal tract of a healthy cat. Phenotypic analysis revealed that *P. vulneris* G3 exhibited a multidrug-resistant (MDR) phenotype, remaining susceptible only to cefoxitin, levofloxacin, and ertapenem. Whole-genome sequencing identified a circular chromosome harboring multiple virulence factors commonly found in the *Enterobacteriaceae* family. Notably, a 300 kb incompatibility group HI2 megaplasmid (pIncHI2A) was detected, carrying a conjugation module and a resistance module with a scrambled structure, reflecting intense activity of mobile elements, such as insertion sequences (e.g., IS*26* and IS*Ecp1*), class I integrons, and degenerated transposons (e.g., a Tn*402*-like element carrying mercury tolerance). The resistance module harbors 15 antibiotic resistance genes (*aac(3)-IIa, aac(6')-Ib10, aph(6)-Id, aph(3")-Ib, ant(3")-Ia*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, $bla_{OXA\text{-}1}$, *sul2*, *qnrB20*, *dfrA14, tet(A), tetR, catB3*, and *catA1*), most of which are associated with these mobile elements. Additionally, genes conferring tolerance to tellurium and arsenic, copper/nickel/cobalt efflux pumps, and a type II HipBA toxin-antitoxin system were identified. Given the relevance to the One Health approach, if *P. vulneris* G3 were to become a zoonotic pathogen, the infections it causes would likely be challenging to treat. This study reports the first identification of a pIncHI2A in *P. vulneris*, revealing the complex genetic structure underlying MDR, and underscores the need for ongoing surveillance of resistance genes across human, animal, and environmental reservoirs.

Impact statement: Antimicrobial resistance poses a significant global public health threat. This study highlights the multidrug-resistant (MDR) nature of *Pseudescherichia vulneris* G3, isolated from a healthy cat's intestinal tract, and its potential implications within the One Health framework. By identifying a unique 300 kb IncHI2 megaplasmid harboring 15 resistance genes and mobile genetic elements, this research emphasizes the pathogen's capacity for genetic plasticity and the risk of zoonotic transmission. The findings underscore the urgent need for comprehensive surveillance of resistance determinants across human, animal, and environmental reservoirs to address the escalating challenge of antimicrobial resistance effectively.

1. Introduction

Pseudescherichia vulneris (*P. vulneris*) is a Gram-negative, oxidase-negative, fermentative, and motile rod from the order *Enterobacterales* and family *Enterobacteriaceae* (Brenner et al., 1982). Initially classified within the *Escherichia* genus, this bacterium was reclassified as *P. vulneris* due to its genetic variability (Alnajar and Gupta, 2017). It is an opportunistic pathogen capable of colonizing various sites in both humans and animals, including wounds and the respiratory, genital, urinary tracts, and feces (Brenner et al., 1982; Alnajar and Gupta, 2017).

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As a result, *P. vulneris* is becoming an important microorganism for both human and veterinary health, as it poses a risk of zoonotic transmission and may contribute to the spread of antimicrobial resistance genes (ARGs) (Shobrak and Abo-Amer, 2014; Zilli et al., 2023).

The indiscriminate use of antimicrobial agents is well known to promote the selection and proliferation of antimicrobial-resistant or multidrug-resistant (MDR) microorganisms, representing a critical public health challenge (Castañeda-Barba et al., 2023). In the context of the One Health approach, companion animals can serve as reservoirs and vectors for ARGs transmission via lateral gene transfer (LGT) mechanisms, contributing to the spread of resistance to humans (Joosten et al., 2020).

Plasmids, along with mobilization elements like insertion sequences (IS), transposons (Tn), and integrons (In), are key vehicles for LGT and the spread of ARGs and other resistance-related genes (Ross et al., 2021). Plasmids are classified into various incompatibility (Inc) groups determined by their replication and partitioning systems (Helinski, 2022). Plasmids within the same Inc group cannot coexist in a single bacterium because they share similar control mechanisms for replication, leading to competition. Conversely, plasmids from different Inc groups can coexist stably within the same cell, facilitating of genetic material between them escalating horizontal transfer (Garcillán-Barcia et al., 2023). Among these, IncHI2 plasmids carrying both ARG for clinically important antibiotics and heavy metal tolerance (HMT) elements have emerged as significant public health concerns due to their conjugative ability and pose a threat in the management of bacterial infections (Algarni et al., 2024). A key factor in plasmid stability is the presence of toxin-antitoxin (TA) systems, which play a vital role in "plasmid addiction." TA systems ensure that daughter cells losing the plasmid during division are eliminated by the toxin, while those retaining the plasmid survive due to the antitoxin. This mechanism preserves the plasmid within bacterial populations, even without selective pressures like antibiotics (Chan et al., 2023).

In association with IS, Tn, and In, plasmids facilitate the acquisition, rearrangement, and dissemination of resistance genes, allowing bacteria to rapidly adapt to selective pressures, such as antibiotic use (Ross et al., 2021). This dynamic interaction accelerates the spread of MDR, posing significant challenges to public health and infection control.

For instance, extended-spectrum β-lactamases (ESBLs), which hydrolyze penicillins, cephalosporins and aztreonam, carbapenemases (bla_{NDM-5}), mobilized colistin resistance (*mcr-like*), and TA systems are widespread in both human and animal bacterial plasmid isolates, in particular IncHI2 plasmids (Algarni et al., 2024; Cain and Hall, 2012; Wang et al., 2022; Tang et al., 2022). The CTX-M family is the predominant ESBL in the *Enterobacterales* order in which *P. vulneris* belongs, with $bla_{CTX-M-15}$ being the most frequently reported ARG in plasmid isolates from dogs and cats in the United States (Woerde et al., 2023).

Despite its potential pathogenic role, certain species within *Pseudescherichia* genus have been reported to exhibit endophytic characteristics, suggesting potential as biocontrol agents against phytopathogens (Gao et al., 2023). However, the discovery of its endophytic nature implies that *Pseudescherichia* may be more widespread than previously thought, raising the risk of ARG transmission via LGT across distinct *Pseudescherichia* species by diverse animal, environmental, and agricultural pathways. This broad distribution heightens concerns about its potential impact on public health.

Given the increasing potential role of *Pseudescherichia vulneris* as a zoonotic pathogen and its capacity to harbor multidrug resistance genes within plasmids, this study aimed to characterize a strain of *P. vulneris* isolated from a healthy cat intestinal tract. By examining its ARG profile, virulence factors, and potential for LGT associated with plasmids and their mobilization elements (IS, Tn, and In), this study provides critical insights into the public health risks posed by this bacterium.

2. Material and methods

2.1. P. vulneris isolate

Pseudescherichia vulneris isolate was obtained from a rectal swab of a healthy puppy cat in Ribeirão Preto, São Paulo State, Brazil, in 2022. Bacterial identification was carried out by MALDI-TOF MS (Matrix Associated Laser Desorption-Ionization – Time of Flight) in a Bruker AutoFlex LT mass spectrophotometer (Bruker Daltonics, Inc.) using the Bruker MALDI BioTyper System (v3.1 Bruker Daltonics, Inc.).

2.2. Antibiotic susceptibility testing

The susceptibility test was performed by agar disk diffusion method to assess the antimicrobial susceptibility of 17 antimicrobials, 7 beta-lactams, amoxicillin/clavulanate 20/10 μg (AMC), cefotaxime 30 μg (CTX), ceftazidime 30 μg (CAZ), cefepime 30 μg (FEP), cefoxitin 30 μg (FOX), aztreonam 30 μg (ATM), ertapenem 10 μg (ETP), 10 non-beta-lactams, gentamycin 10 μg (GEN), tobramycin 10 μg (TOB), amikacin 30 μg (AMK), enrofloxacin 5 μg (ENR), nalidixic acid 30 μg (NAL), ciprofloxacin 5 μg (CIP), levofloxacin 5 μg (LEV), tetracycline 30 μg (TET), sulfamethoxazole/trimethoprim 1,25/23,75 μg (SXT) and chloramphenicol 30 μg (CLO), following the Clinical and Laboratory Standards Institute (CLSI) recommendation. Minimal inhibitory concentration (MIC) was performed by Etest® (bioMerieux) and by the reference broth microdilution method in a 96-well plate according to the CLSI guidelines, and the breakpoints were evaluated according to the CLSI (CLSI. CLSI, M100-ED33, 2023). MIC was carried out for the antibiotics that showed a resistance profile by the agar disk diffusion method. The isolate showed an ESBL-producing phenotype by double disk synergism using cefotaxime, ceftazidime, cefoxitin, and aztreonam plus amoxicillin/clavulanic acid.

2.3. Polymerase chain reaction (PCR) for β-lactamase genes identification

PCR was employed to identify the presence of β-lactamase genes (e.g., $bla_{CTX-M-GROUP-1}$, $bla_{CTX-M-GROUP-2}$, $bla_{CTX-M-GROUP-8}$, and $bla_{CTX-M-GROUP-9}$, bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{GES}, bla_{BEL}, and bla_{VEB}). DNA was extracted following instructions previously described (Bolano et al., 2001). The extracted DNA was quantified using a Nanodrop spectrophotometer and adjusted to a final concentration of 60 ng/μL for PCR reactions. The primers used and PCR conditions are listed in Table S1.

2.4. S1 endonuclease pulsed-field gel electrophoresis (PFGE)

To determine the plasmid content, *S1*-PFGE was performed following standard protocols (Barton et al., 1995). Bacterial cells were first embedded in low-melting-point agarose plugs to preserve their DNA structures. The presence of plasmids was determined by PFGE after digestion with *S1* nuclease and analyzed in PFGE gel (*S1*-PFGE) performed on the CHEF DRIII system (Bio-Rad, USA) (Ribot et al., 2006).

Plasmid sizes were estimated by comparison to a molecular weight marker (*Salmonella* serotype Braenderup strain H9812) (Hunter et al., 2005). The results provided an estimate of the number and size of the plasmids harbored by the isolate.

2.5. Conjugation assay

The conjugation assay was performed using *Escherichia coli* J53 (sodium azide-resistant) as the recipient strain and *P. vulneris* G3 as the donor. *E. coli* J53 transconjugants were selected on MacConkey agar containing 2 μg/mL of cefotaxime (CTX) and 100 μg/mL of sodium azide, as previously described (Dionisio et al., 2002).

2.6. *P. vulneris* whole-genome sequencing (WGS) by short, and long-reads

WGS was performed using Illumina MiSeq for short reads (2 ×250 bp, with an average depth coverage of approximately 100 ×) and Oxford Nanopore MinION for long reads. Genomic DNA was extracted from fresh *P. vulneris* isolate cultures grown in BHI broth (KASVI®) using the Maxwell® RSC Cultured Cells DNA Kit (Promega). DNA quality was assessed using a NanoDrop™ spectrophotometer, focusing on the 260/280 ratio, while DNA concentration was measured with the Qubit dsDNA HS Assay Kit and a Qubit 4 fluorometer (Thermo Fisher Scientific).

A paired-end library was prepared using the Illumina DNA Library Prep Kit for the Illumina sequencing, following the manufacturer's protocols. Fragment lengths were analyzed using the TapeStation 2200 (Agilent, UK) to ensure proper size distribution.

In addition, Oxford Nanopore MinION sequencing was performed using an R9.4 flow cell, achieving approximately 100 × coverage. DNA extraction for the MinION sequencing was carried out with the QIAamp® DNA Mini Kit (QIAGEN), and the sequencing library was prepared using the Rapid Barcoding Sequencing Kit (SQK-RBK004; Oxford Nanopore), according to the manufacturer's instructions.

2.7. Genome assembly, annotation, and analysis

The genome was assembled using SPAdes v3.15.5 (Bankevich et al., 2012). The assembled genome was reoriented and circularized using custom scripts. The genome annotation was carried out with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). Average Nucleotide Identity was calculated using the "ani.rb" script from the enveomics package (Rodriguez-R and Konstantinidis, 2016) against *P. vulneris* strain 4928STDY7071515, the Genbank reference genome (NZ_LR607338.1). The genome completeness was evaluated with CheckM v1.2.2 (Parks et al., 2015).

Furthermore, the Center for Genomic Epidemiology (CGE) (https://www.genomicepidemiology.org/) tools ResFinder 4.6 (Florensa et al., 2022), VirulenceFinder 2.0 from Virulence Factor Database (VFDB) (Chen et al., 2005), and PlasmidFinder 2.1 (Carattoli et al., 2014) were used to identify resistance genes, virulence determinants, and plasmid replicon types. Prophages were determined with the PHASTER platform (Arndt et al., 2016).

IS, Tn, In, ARG, and HMT genes were manually annotated using TnCentral (Ross et al., 2021) annotation procedures and custom library using the SnapGene software (https://www.snapgene.com/). The figures were generated with SnapGene and manually edited with Inkscape (https://inkscape.org/). The graphic abstract was created with BioRender.com.

2.8. Data availability

The complete assembled and annotated genome, and raw reads were deposited in the GenBank, under the BioProject PRJNA1145325.

3. Results and discussion

3.1. Genetic and phenotypic characterization of Pseudescherichia vulneris G3

According to the MALDI-TOF MS analysis, the isolate was identified and classified as *P. vulneris* G3, with a score of 2.13, indicating high confidence in the identification at the species level. Antimicrobial susceptibility testing revealed an MDR phenotype, defined as non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012). The isolate remained susceptible only to cefoxitin, levofloxacin, and ertapenem (Table 1). Additionally, the presence of β-lactamases genes was investigated by PCR, which confirmed the presence of $bla_{\text{CTX-M-GROUP-1}}$ (Fig. S1). *S1*-PFGE analysis revealed the presence of two plasmids: a smaller one approximately 30 Kb in size, and a larger one ranging between 280 and 300 Kb (Fig. S2).

Table 1
In vitro evaluation of antimicrobial activity against *P. vulneris* G3 strain.

Antimicrobial[a]	Susceptibility profile[b]	MIC (μg/mL)[c]
AMC	R	64
CTX	R	≥256
CAZ	R	16
CPM	R	≥16
FOX	S	-
ATM	R	12
ETP	S	-
GEN	R	≥16
TOB	I	18
AMK	R	11
ENR	I	-
NA	I	-
CIP	I	-
LVX	S	-
TET	R	≥256
SXT	R	≥32
CHL	R	≥256

[a] AMC, amoxicillin-clavulanate, CTX, cefotaxime, CAZ, ceftazidime, CPM, cefepime, ATM, aztreonam, ETP, ertapenem, GEN, gentamicin, TOB, tobramycin, AMK, amikacin, ENR, enrofloxacin, NA, nalidixic-acid, CIP, ciprofloxacin, LVX, levofloxacin, TET, tetracycline, SXT, sulfamethoxazole-trimethoprim, CHL: chloramphenicol.
[b] R, resistant; S, susceptible; I, intermediate
[c] MIC, minimal inhibitory concentration; - not determined

Further genome sequencing using a hybrid approach revealed a main circular chromosome of 4,5 Mb and confirmed the two identified plasmids (Table 2), consistent with the genome size of other *P. vulneris* strains deposited in the GenBank database. Analysis by ANI and CheckM revealed 96.35 % (SD of 1.67 %) of ANI, 98.47 % of genome completeness, 0.81 % contamination, and 0 % of strain heterogeneity (Table 2).

3.2. *P. vulneris* G3 general genomic features

According to the VFDB and genomic annotation, *P. vulneris* G3 harbored several virulence determinants commonly found in the family *Enterobacteriaceae* (Azam et al., 2023) that equip the bacterium to effectively colonize hosts, evade immune defenses, and establish infections (Table S2). For instance, curli fibers (*csgA* and *csgC*), are involved in surface adhesion, cell aggregation, and biofilm formation.

Table 2
Genomic features of *P. vulneris* G3 strain.

Data	*P. vulneris* G3
Sequencing reads	
Illumina (2x250bp)	1212,273
ONT	19,606 (N50 of 8.6 kb)
Genome size	
main chromosome	4516,683 bp
plasmid pIncHI2A	300,111 bp
plasmid2	30,524 bp
Average Coverage	115x
GC	55.49 %
Genes (total)	4777
CDSs (total)	4668
Genes (coding)	4588
Genes (RNA)	109
rRNAs	7, 6, 6 (5S, 16S, 23S)
tRNAs	79
ncRNAs	11
Pseudo Genes (total)	80
CRISPR Arrays	1
Prophage regions	7

The strain also carried *E. coli* hemorrhagic pilus (HCP) genes (*hcpA* and *hcpC*), known to induce pro-inflammatory cytokine secretion in intestinal epithelial cells. Genes involved in adherence such as to lateral flagellum (*flgC*), flagellum group I (*fliC*, *fliD*, and *fliS*), type IV pili (*pilW*), Streptococcal plasmin receptor/GAPDH (*gapA*), and fimbrial adherence determinants including *Salmonella* peg (*pegB*) - related to SEF14 fimbriae gene cluster - and *Salmonella stcC* (similar to *fimD*) were detected. Moreover, aerobactin siderophore (*iutA*), pyoverdine (*pvdH*), and enterobactin siderophore (Ent) associated with iron uptake system, brain endothelial cell invasion (*ibeC*), LPS glycosylation (*gtrA and gtrB*), O-antigen (*cpsB*), and polysaccharide poly-N-acetylglucosamine (*pgaC*) – involved in biofilm formation - were described. Furthermore, genes related to the types IV and VI secretion systems were detected. These virulence determinants reflect the complex arsenal employed by *P. vulneris* G3, highlighting its potential to cause significant disease, particularly if zoonotic transmission to humans were to occur.

Additionally, a total of seven intact prophage regions were identified (Fig. S3). It is well established that prophages can significantly influence the lifestyle, fitness, virulence, and evolutionary trajectory of their bacterial hosts in numerous ways (Fortier and Sekulovic, 2013). Therefore, this finding supports the hypothesis that the main chromosome is currently undergoing active LGT and exhibiting genomic plasticity, potentially enhancing the adaptability and resilience of *P. vulneris* G3. Interestingly, no ARG and few IS were identified in the *P. vulneris* G3 main chromosome.

The first plasmid is small, with a size of 30 kb, encoding a few genes related to conjugation, a toxin-antitoxin system associated with plasmid stability, and several hypothetical and uncharacterized genes (Fig. S4). No ARG and HMT genes were identified.

The second plasmid is larger, spanning 300 kb, and is therefore characterized as a megaplasmid (Fig. 1). PlasmidFinder and genome annotation identified the presence of the IncHI2/IncHI2A plasmid replicon type and associated genes, which belong to ST01 (pMLST) (Carattoli et al., 2014). IncHI2A plasmids are known to be larger plasmids that are widely distributed among *Enterobacteriaceae*, particularly in MDR *Salmonella* strains (Algarni et al., 2024). As previously reported for IncHI2 plasmids (Algarni et al., 2024), P. vulneris G3 pIncHI2A also contains 25 IncHI2-associated conjugal transfer genes. To date, this is one of the first reports of an IncHI2A plasmid in *Pseudescherichia vulneris*, making this one of the earliest documented instances of an IncHI2A

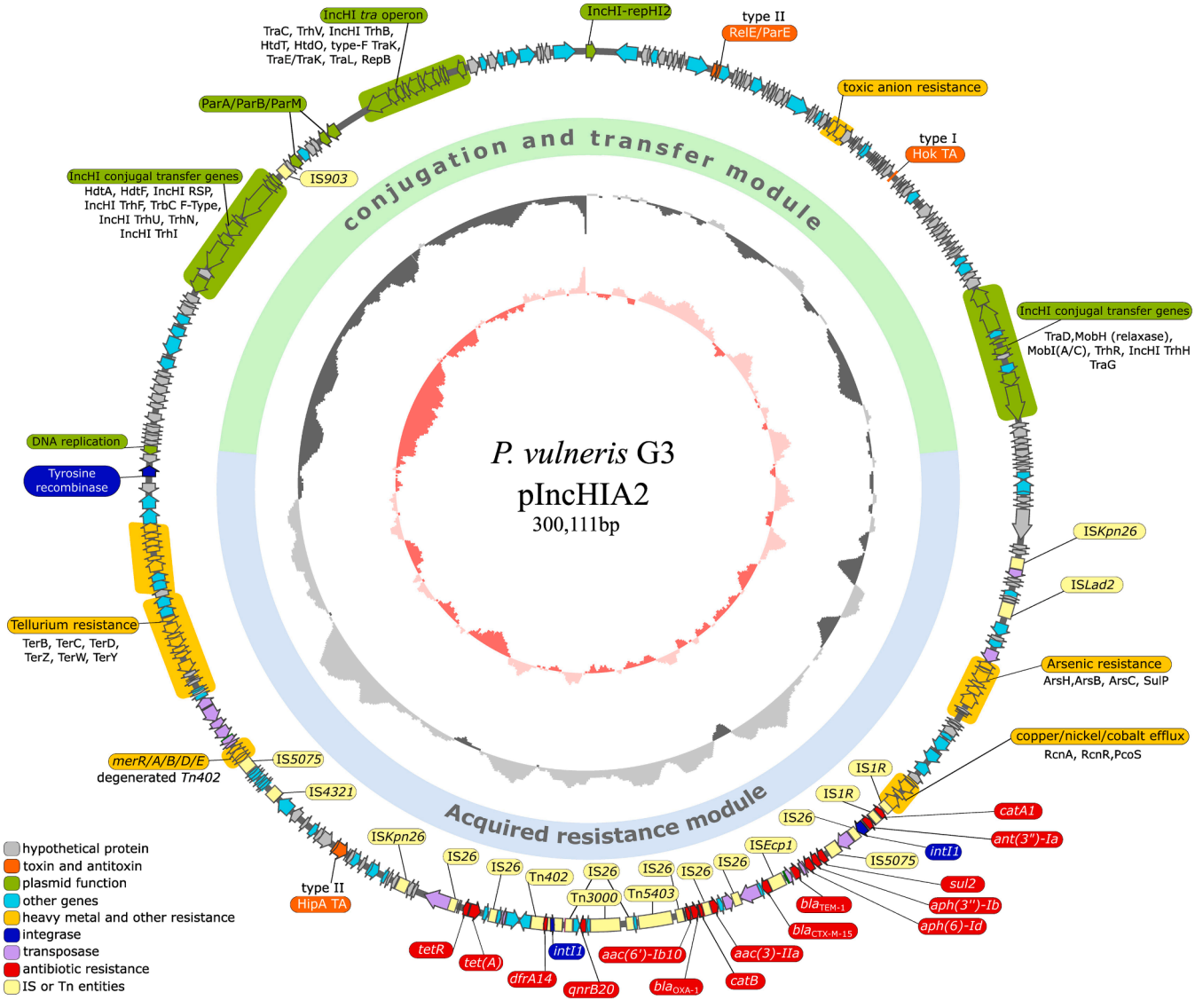

Fig. 1. Genomic representation of *Pseudescherichia vulneris* G3 IncHI2A plasmid. Gene orientations are represented by arrows and are color-coded according to their function, as shown in the legend. The outermost circle represents the conjugation, replication and transfer, and acquired resistance modules. The internal circles display the GC content and GC-skew, respectively.

plasmid in *Pseudescherichia vulneris*.

Despite the presence of complete conjugation machinery, our in vitro conjugation assay revealed no successful transfer of the megaplasmid. One possibility is that the plasmid's substantial size (~300 kb) may hinder its transfer under standard laboratory conditions, as larger plasmids sometimes require specific host factors or alternative conditions for successful mobilization (Thomas and Nielsen, 2005). Additionally, although other IncHI2A plasmids are well known to be conjugative and to spread among *Salmonella* and other *Enterobacteriaceae* (Carattoli, 2009), the non-conjugative result in this study does not necessarily rule out the possibility of transfer under different experimental settings or in different host strains.

Similarly to other IncHI2A plasmids, *P. vulneris* G3 pIncHI2A harbors two distinct modules: a replication, conjugation and transfer module, and an acquired resistance module, which are distinguishable by their distinct GC content and GC-Skew patterns (Fig. 1). The conjugation and transfer module exhibits a GC content of approximately 43 %, while the acquired resistance module presents a GC content of 50 %. The differences in GC% and GC-skew patterns suggest that these modules may have followed distinct evolutionary trajectories during the assembly of this plasmid, reflecting the potential acquisition of genetic material from different sources through LGT (Frost et al., 2005).

Furthermore, while the replication, conjugation and transfer module contain a type II TA system belonging to the RelE/ParE superfamily and a hok-sok type I TA, the acquired resistance module features a HipBA type II TA system. These TA systems are involved in plasmid addiction, ensuring that plasmid-free daughter cells are eliminated, thereby maintaining the plasmid within the bacterial population (Hayes, 2003). Notably, the HipBA TA system induces a dormant, persisted cell state by inhibiting protein synthesis, allowing bacterial cells to temporarily survive antibiotic treatment without developing genetic resistance (Keren et al., 2004), thereby contributing to the persistence of *P. vulneris* G3 infections and making them more difficult to eradicate.

3.3. *Pseudescherichia vulneris G3 pIncHI2A heavy-metal tolerance and antibiotic-resistance features*

The replication, conjugation and transfer module carry two toxic anion tolerance genes, TelA (IPR008863), which may provide the bacteria with a first line of defense to survive and grow in the presence of harmful, negatively charged ions. Toxic anions, such as tellurium (TeO_3^{2-}), arsenate (AsO_4^{3-}), and selenate (SeO_4^{2-}), can disrupt essential biochemical pathways by interfering with enzymes, redox reactions, and other cellular functions (Chasteen et al., 2009).

It is interesting that a tyrosine recombinase (integrase) gene, core-binding (CB) (IPR044068) and catalytic integrase domains (IPR002104), are found at the boundary between the conjugative and resistance modules (Fig. 1). It is possible that this has played a role in the assembly of the plasmid (Smyshlyaev et al., 2021). For instance, the resistance module contains a number of heavy metal tolerance genes. There are two divergent sets of the tellurium resistance genes one of which contains seven genes (*terB*, *terC*, 3x *terD*, *terF* , *terZ*), and the other with five genes (*terD*, 2x *terY*, *terY-C*, *terW*), both located upstream of this integrase (Fig. 2A).

Additionally, mercury (Hg^{2+} toxic cation) resistance genes (*merA*, *merB*, *merD*, *merE*, and *merR*) are embedded within a structure resembling a Tn*402* family element (Fig. 2B). This Tn*402*-like structure encodes a full set of transposition-related genes including a resolution site and carries an insertion of IS*5075* from IS*110* family. It is bounded by two IRt copies instead of one IRt and one IRi and does not have direct target repeats. This configuration might suggest that the structure has lost some of its transposition functionality, potentially stabilizing the resistance genes within the genome, while still maintaining their expression under selective pressure. The final set of HMT genes is related to copper, nickel, and cobalt efflux pumps, as well as arsenic tolerance genes (Fig. 1). No clear association with IS or Tn was identified for these genes, making it difficult to predict the acquisition pathway.

There is a total of 15 ARG — *aac(3)-IIa, aac(6')-Ib10, aph(6)-Id, aph(3")-Ib, ant(3")-Ia,* $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, $bla_{OXA\text{-}1}$, *sul2, dfrA14, qnrB20, tet(A), tetR, catB3*, and *catA1*— conferring resistance to aminoglycosides, beta-lactams, sulfonamides, quinolones, tetracyclines, and phenicol (Fig. 1). The genes identified confer resistance to the main antibiotics used in both human and veterinary clinic, being the beta-lactams the first-line antibiotic therapy for infections caused by Gram-negative bacteria (Joosten et al., 2020). This extensive resistome corroborates the strain's MDR phenotypic profile, posing significant challenges for treatment options.

Interestingly, a comparative analysis based on a previous survey of 667 IncHI2 plasmids deposited in GenBank (Algarni et al., 2024) revealed that among the HMT genes identified, the tellurium tolerance genes (*terB, terC*, and *terY*), the nickel/cobalt efflux system genes (*rcnA, rcnR*), and the arsenic tolerance gene (*arsH*) were exclusive for *P. vulneris* G3 pIncHI2A. Additionally, along with the rare occurrence of *aac(3)-IIa* and $bla_{CTX\text{-}M\text{-}15}$ in IncHI2 plasmids, the genes *aac(6')-Ib10, ant(3")-Ia,*

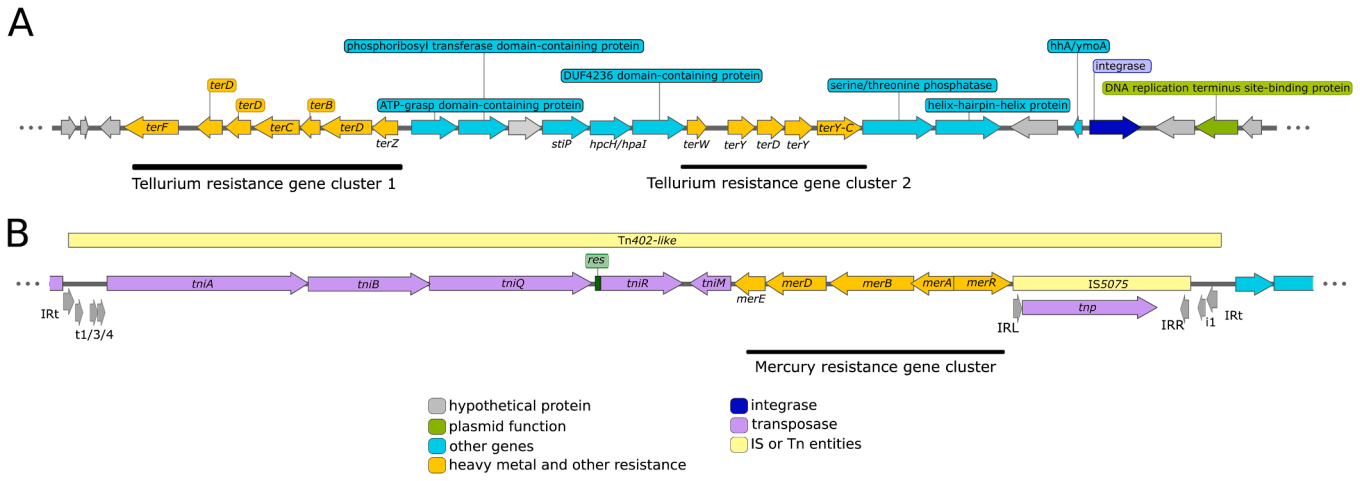

Fig. 2. Genetic context of the tellurium (A) and mercury (B) tolerance genes in *P. vulneris* G3. Gene orientations are represented by arrows and are color-coded according to their function, as shown in the legend. (A) Tellurium tolerance genes cluster 1 and 2 are located upstream of an integrase. (B) Mercury tolerance genes are embedded within a Tn*402*-like element. Notably, this Tn*402*-like element does not exhibit the typical ends expected in such elements, which should contain short ~19–25 bp inverted repeats (IR) at their ends, specifically IRt (proximal to transposase) and IRi (proximal to integron integrase). Additionally, this element typically generates a 50-bp direct repeat (DR) upon insertion. These features (IRi and DR) are absent.

bla$_{TEM-1B}$, *qnrB20*, and *tetR* were also found exclusively in *P. vulneris* G3 pIncHI2A.

3.4. Genetic architecture of ARGs and their association with Tn, IS, In, and LGT in P. vulneris G3 pIncHI2A

The ARGs were associated with various chimeric structures involving Tn, In, and IS (Fig. 3A-F). Although the exact assembly of most of these structures remains unclear, the origins and potential mobilization activity of some can be relatively well understood.

For instance, *dfrA14* is part of an integron cassette (class I integron) carried by an incomplete Tn*402* family transposon, which includes its own integrase gene (Fig. 3A). The integron carries a promoter, ensuring the expression of *dfrA14* and conferring the observed resistance to trimethoprim. This configuration with the integrase gene, the integron promoter, and a typical *attI*, suggests that the integron is still functional in capturing and expressing resistance genes, despite the incomplete nature of the Tn*402*, and highlights the evolutionary persistence of this gene cassette in maintaining antimicrobial resistance under selective pressure. Moreover, as previously observed in clinically relevant class I integrons (Betteridge et al., 2011), this integron cassette is likely to be mobilized in trans using the intact transposition machinery of the other Tn*402*-related element carrying mercury resistance (Fig. 2A). The incomplete Tn*402* element presents the IRs and DRs and lacks four essential transposition-related genes: TniA, TniB, TniQ, and TnsD/TniQ. Specifically, the *tniA* gene has been disrupted by an IS*6100* element from the IS*6* family. Interestingly, this incomplete Tn*402* element also carries a relaxase with a *mobC* domain (IPR008687), which facilitates plasmid mobilization through conjugation (Zhang and Meyer, 1997). Although *P. vulneris* possesses its own IncHI relaxase (*mobH*) gene, the presence and potential role of this acquired *mobC* gene with the plasmid fitness remains to be established (Fig. 3A).

aac(6′)-Ib10 and its neighbor *bla*$_{OXA-1}$ are embedded in type I integron cassettes that lack their own integrase. Alongside the *catB* gene, they form a contiguous structure, similar to integrons like In*37* (Wang et al., 2003), and are flanked by inverted IS*26* copies (Fig. 3B). This arrangement suggests that these genes likely originated from a functional integron and were later mobilized into the current genomic context through recombination events. The absence of the integrase implies that further acquisition or movement of additional gene cassettes may not be possible in this configuration, limiting further integration while ensuring the continuous expression of these resistance genes under selective pressure. Conversely, the *aac(3)-IIa* gene is located within an two directly repeated IS*26* copies, suggesting the formation of a potential pseudo-compound transposon structure. However, these arrangement lacks the characteristic ~8 bp DRs typically required for transposition activity (Varani et al., 2021). This absence of DRs suggests that *aac(3)-IIa* gene may have originally been mobilized by an IS*26*-mediated compound Tn transposition event (Fig. 3B).

The *ant(3")-Ia* gene is clearly associated with a class I integron (with the expected recombination sites) and is located upstream of an integron integrase gene that contains a LexA binding site, which is associated with host control by the S.O.S. response system (Guerin et al., 2009) (Fig. 3C). This suggests that the expression and mobilization of this gene cassette may be regulated by the host's S.O.S. response, allowing the bacteria to respond to DNA damage or stress by activating integrase-mediated recombination events, thereby facilitating the capture and dissemination of antibiotic resistance genes. The *catA1* gene and its promoter are located between two inverted IS*1*R copies. However, no flanking 8/9 bp direct target repeats are observed, making it difficult to determine whether this configuration represents a true compound transposon (Fig. 3C), or if *catA1* was acquired through an earlier event involving a complete and functional compound transposon. The absence of target site duplications suggests that any previous transposition activity might have degenerated, complicating the reconstruction of its acquisition history.

The *qnrB20* gene is situated between two directly repeated IS*26* copies that lack the characteristic ~8 bp DRs (Varani et al., 2021) indicating that the acquisition of *qnrB20* likely occurred via a previous IS*26*-mediated compound transposon. However, the absence of DRs suggests that this structure may no longer retains active transposition capability, making the current configuration static and unable to mobilize further (Fig. 3D).

The *bla*$_{CTX-M-15}$-IS*Ecp1* module is inserted within a Tn*3*-like transposon between the transposase (*tnpA*) gene and the resolution site (*res*); the *bla*$_{TEM-1B}$ gene is also part of this decayed Tn*3* transposon, which includes both the *tnpA* and resolvase (*tnpR*) genes, as well as the res site and an inverted repeat left (IRL) typical of Tn*3* (see for example: (Varani et al., 2024)), but lacks the inverted repeat right (IRR) (Fig. 3E). As is commonly observed, *bla*$_{CTX-M-15}$ is associated with IS*Ecp1* (Awosile and Agbaje, 2021), and its expression is driven by an IS*Ecp1*-embedded promoter. *bla*$_{TEM-1B}$ also exhibits its own promoter. Together, the promoters for *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1B}$ ensure the expression of both genes, thereby confirming the observed MDR phenotype. However, due to the absence of essential elements like the IRR, the decayed Tn*3* structure would lack mobility, leaving these resistance genes fixed within *P. vulneris* G3 pIncHI2A.

The *aph(6)-Id* and *aph(3'')-Ib* genes, along with a Tn*3*-family IRR, are present in Tn*5393*, which is commonly found in *Erwinia amylovora* and other Gram-negative bacteria (Chiou and Jones, 1993). These sequences, including *sul2*, are also found in Tn*7114* (Muñoz-Gutiérrez et al., 2024). Thus, the genetic context of *aph(6)-Id, aph(3'')-Ib*, and *sul2* in *P. vulneris* G3 pIncHI2A (Fig. 3E) suggests a potential acquisition pathway related to Tn*5393* and Tn*7114* relatives, now appearing as decayed structures in the current arrangement, indicating that they may no longer retain active transposition capability.

The last two resistance genes, *tetA*, and *tetR*, are found associated with a degenerated Tn*3* family element that retains the *tnpR* and *tnpA* genes but lacks the left IR and both DRs. Notably, *tetA* and *tetR* genes were originally observed in Tn*1721*, a member of the Tn*3* family (Schmitt et al., 1979), suggesting that the original acquisition pathway of these genes in *P. vulneris* G3 pIncHI2A might be related to a Tn*1721* derivative. Furthermore, both *tetA* and *tetR* genes are intersected by flanking inverted IS*26* copies (Fig. 3F), indicating a possible involvement of IS*26* in their mobilization. However, this arrangement, lacking critical transposition elements, suggests the structure may no longer be capable of active transposition, leaving the resistance genes in a fixed in pIncHI2A position.

These findings indicate that the acquired resistance module presents a mosaic of incomplete transposable elements (Tn, In, and IS), harboring multiple ARGs. Notably, many of these transposition-related elements lack DRs and IRs, which likely impedes active transposition. However, the presence of *attC* sites in genes such as *bla*$_{OXA-1}$ and *aac(6′)-Ib10* suggests that some level of mobility could still be facilitated via integrase-mediated mechanisms. Overall, these findings indicate a history of gene acquisition through recombination and transposition, followed by partial functional decay, resulting in a stable module that retains its ARG repertoire yet shows limited potential for further rearrangement under normal conditions.

4. Conclusions

In this study, we provided the first phenotypic and genomic characterization of *Pseudescherichia vulneris* G3, identifying the presence of an IncHI2A megaplasmid within this species for the first time. This plasmid, with its distinct architecture and evolutionary trajectory, serves as a reservoir for 15 ARGs and several HMT genes. These resistance determinants, including those for third- and fourth-generation cephalosporins, tellurium, mercury, and arsenic, were found embedded within an array of IS, In, and Tn, highlighting the significant role of LGT in their acquisition and assembly. The structural complexity of the acquired resistance module, characterized by a scrambled mosaic

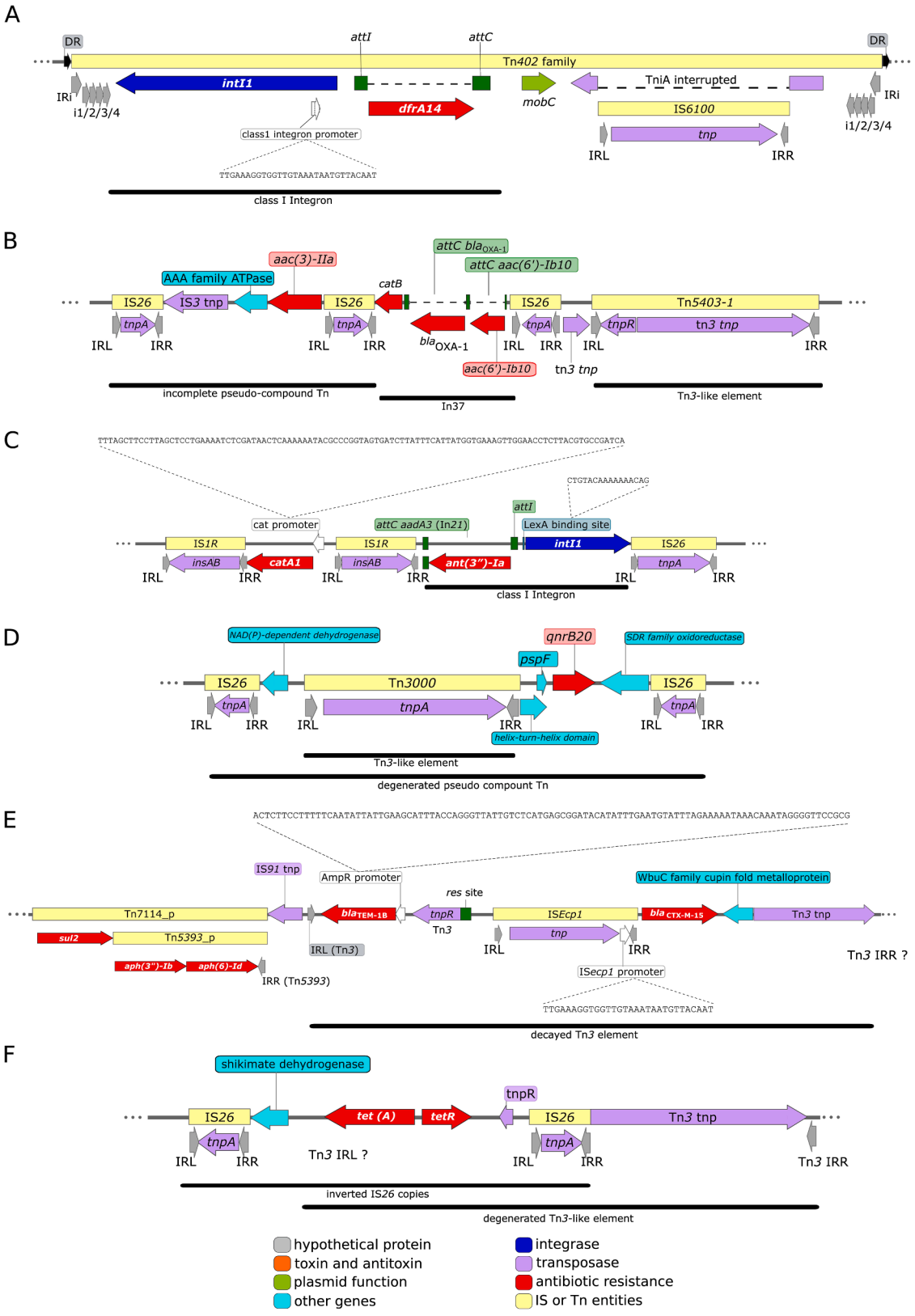

Fig. 3. Genetic context of all ARGs identified in *P. vulneris G3*. The genetic context of the antimicrobial resistance genes (ARGs) is shown across six panels (A–F). Gene orientations are represented by arrows and are color-coded according to their function, as shown in the legend. (A) *dfrA14* gene. (B) *aac(3)-IIa, catB,* bla_{OXA-1}, and *aac(6')-Ib* genes. (C) *catA1* and *ant(3")-Ia* genes. (D) *qnrB20* gene. (E) *sul2, aph(3")-Ib, aph(6)-Id,* bla_{TEM-1}, and $bla_{CTX-M-15}$ genes. The "_p" suffix in Tn*7114_p* and Tn*5393_p* indicates that these elements are partial Tn. This means that they do not contain all the genes or functional sequences required for autonomous transposition or complete functionality. (F) *tetA* and *tetR* genes.

of genetic elements, reflects a history of frequent gene transfer events, contributing to its stability but limiting further mobility.

Moreover, the presence of multiple TA systems, particularly the HipBA system, suggests that *P. vulneris* G3 could be more challenging to treat, as these systems may promote the survival of persister cells during antibiotic treatment. This persistence could lead to recurrent infections, complicating treatment outcomes.

Interestingly, the presence of curli fibers, flagella, and various pili involved in adhesion and biofilm formation, combined with siderophores for iron uptake and heavy metal tolerance genes, suggests that *P. vulneris* G3 is well-suited for survival in both host tissues and diverse environmental niches. These factors support the hypothesis that *P. vulneris* G3 may be also widespread across different environments, posing a risk not only to animal health but also to human health through potential zoonotic transmission.

Finally, the presence of an integrase and integron structures in pIncHI2A, along with evidence of active LGT in *P. vulneris* G3 main chromosome, as revealed by the presence of intact prophage regions, may suggest ongoing genetic mobility and the potential for further acquisition and dissemination of ARG and virulence factors. This combination of mobile genetic elements and prophage activity highlights the evolutionary capacity of this strain to adapt and pose increasing challenges to both animal and human health.

Indeed, this is not the first instance of *P. vulneris* being identified in domestic animals—previous studies have reported its presence in a skin lesion of an ill domestic cat (Zilli et al., 2023), raising the hypothesis that bacteria from this genus may affect domestic animals. Even though recent epidemiological surveillance studies suggest that interactions between humans and their pets have a minimal impact on the spread of multidrug-resistant organisms (Genath et al., 2024), the risk of transmission, including LGT of ARGs from a shared source still persists.

Taken together, our findings highlight the urgency of continued research and surveillance of *P. vulneris* strains across different reservoirs. As the world grapples with the antimicrobial resistance crisis, the adoption of a One Health approach is critical to monitoring and mitigating the spread of such pathogens across species and environments. The presence of virulence and resistance determinants in this strain illustrates the complexity of addressing zoonotic threats, where integrated efforts across human, animal, and environmental health are essential. Ongoing research is vital to understanding the broader implications of *P. vulneris* in human, veterinary, and environmental contexts.

Ethics

The study was approved by the Ethics Committee for the Use of Animals of the Ribeirão Preto School of Pharmaceutical Sciences (CEUA FCFRP) n: 24.1.73.60.7.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2025.100268.

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