

Molecular and Functional Characterization of a Novel Plasmid-Borne *bla*_{NDM}-Like Gene, *bla*_{AFM-1}, in a Clinical Strain of *Aeromonas hydrophila*

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Purpose: An increasing frequency of antibiotic resistance has been observed in both clinical and environmental *Aeromonas hydrophila* isolates in recent years. However, there are still very few in-depth studies regarding the role of plasmids in the antibiotic resistance of *A. hydrophila*. Hence, we investigated the molecular and functional characterization of a multidrug-resistant plasmid encoding an NDM-like metallo- β -lactamase, AFM-1, in the clinical *A. hydrophila* isolate SS332.

Methods: The minimum inhibitory concentrations (MICs) of 24 antibiotics against *A. hydrophila* SS332 were measured by the agar dilution method. The genome of *A. hydrophila* SS332 was sequenced with PacBio and Illumina platforms. Six plasmid-borne antimicrobial resistance genes were chosen for cloning, including *bla*_{AFM-1}, *bla*_{OXA-1}, *msr*(E), *mph*(E), *aac*(6')-Ib10, and *aph*(3')-Ia. Phylogenetic analysis, amino acid sequence alignment, and comparative genomic analysis were performed to elucidate the active site requirements and genetic context of the *bla*_{AFM-1} gene.

Results: *A. hydrophila* SS332 showed high levels of resistance to 15 antibiotics, especially those with MIC levels at or above 1024 μ g/mL, including ampicillin, ceftazidime, ceftazidime, aztreonam, spectinomycin, and roxithromycin. Six plasmid-borne resistance genes from *A. hydrophila* were verified to be functional in *E. coli* DH5 α . AFM-1 shared 86% amino acid identity with NDM-1 and showed resistance to ampicillin, ceftazidime, cefoxitin, and ceftazidime. In addition, the *bla*_{AFM-1} gene was associated with three different novel ISCR19-like elements, designated ISCR19-1, ISCR19-2 and Δ ISCR19-3, which may be involved in the acquisition and mobilization of the *bla*_{AFM-1} gene.

Conclusion: Our investigation showed that plasmid-borne resistance genes can contribute to antibiotic resistance in *A. hydrophila* SS332. A novel *bla*_{NDM}-like gene, *bla*_{AFM-1}, was verified to be functional and associated with novel ISCR19-like elements. This fact indicated the risk of spread of *bla*_{AFM-1} genes and ISCR19-like elements.

Keywords: *Aeromonas hydrophila*, whole-genome sequencing, plasmid-borne resistance genes, *bla*_{AFM-1}, ISCR19-like elements

Introduction

Aeromonas hydrophila is a gram-negative bacterium that is ubiquitous in aquatic environments and can cause infections in fish,¹ amphibians,² reptiles,³ and humans.⁴ It has been identified as an antibiotic-resistant and virulent etiologic agent in an increasing list of human diseases, including gastroenteritis,⁵ diarrhea,⁶ necrotizing fasciitis,⁷ septicemia,⁸ meningitis,⁹ and hemolytic uremic syndrome.¹⁰ Unfortunately, clinical and environmental isolates of *A. hydrophila* are becoming increasingly resistant to

multiple antibiotic agents, including resistance to colistin and carbapenem, which are considered as the last line of defense against multidrug-resistant infections.

Hughes et al reported that an *A. hydrophila* AHNIH1 carrying the *bla*_{KPC-2} gene isolated from perirectal surveillance culture was resistant to ertapenem.¹¹ Similarly, *A. hydrophila* GSH8-2 carrying the *bla*_{KPC-2} gene with reduced susceptibility to all tested β -lactams was isolated from wastewater treatment plant effluent in Japan.¹² Interestingly, the abovementioned antimicrobial resistance genes (ARGs) are all located on plasmids in *A. hydrophila*.¹²

Antimicrobial resistance is a global public health challenge that threatens human health. The increasing incidence of phenotypic resistance to antibiotics is mainly attributed to abuse of antimicrobials and acquisition of ARGs by bacterial pathogens.¹³ Several phenotypic properties of bacteria have been proven to be encoded on plasmids, such as antimicrobial resistance and virulence factors.¹⁴ In addition, the dissemination of ARGs is closely associated with mobile genetic elements, including plasmids, which increase their ability to replicate and contribute to self-transmission or horizontal gene transfer among different bacterial species. It has been reported that *A. hydrophila* is able to harbor one or more plasmids. However, there are still very few in-depth studies regarding the role of plasmids in the antibiotic resistance of *A. hydrophila*.

In this study, the multidrug-resistant clinical *A. hydrophila* strain SS332 was isolated from a fecal sample from a male gastric carcinoma patient in Lishui Central Hospital, Zhejiang, China. Genome sequencing showed that the plasmid pSS332-218k from SS332 carries 12 putative ARGs, including *bla*_{AFM-1}, which is a *bla*_{NDM}-like gene with 86% identity to *bla*_{NDM-14}. The metallo- β -lactamase gene *bla*_{AFM-1} was first cloned, and its antibiotic resistance profile was verified. Phylogenetic analysis, amino acid sequence alignment, and comparative genomic analysis were performed to elucidate the active site requirements and genetic context of the *bla*_{AFM-1} gene.

Materials and Methods

Bacterial Strains and Plasmids

A. hydrophila SS332 was isolated from a fecal specimen from a 76-year-old male patient in Lishui Central Hospital, Zhejiang, China in 2013. It was identified by a microorganism autoanalysis system (VITEK[®]2, BioMérieux, France), and homologous comparisons of

the 16S rRNA gene sequence to the GenBank database were performed by using the BLAST program (www.ncbi.nlm.nih.gov/BLAST/). *Escherichia coli* ATCC 25922 was used as the quality control strain in antimicrobial susceptibility testing. *E. coli* strain DH5 α was used as a recipient for cloning experiments. pUCP20 and pUCP24 were used as vectors in cloning experiments.

Antimicrobial Susceptibility Testing

The agar dilution method was employed to measure the minimum inhibitory concentration (MIC) of antibiotics against *A. hydrophila* SS332 according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, M45 3rd edition (2015). Twelve β -lactam antibiotics (ampicillin, cefminox, cefazolin, cefoxitin, ceftazidime, cefotaxime, ceftriaxone, cefoselis, cefepime, aztreonam, imipenem, and meropenem), four aminoglycoside antibiotics (spectinomycin, gentamicin, kanamycin, and amikacin), two macrolide antibiotics (erythromycin and roxithromycin), two phenicol antibiotics (chloramphenicol and florfenicol), three quinolone antibiotics (nalidixic, ciprofloxacin and levofloxacin) and polymyxin B were tested. *E. coli* ATCC 25922 was used as the quality control strain. The results were interpreted according to the standards of the CLSI.

Whole-Genome Sequencing and Bioinformatic Analysis

Genomic DNA of *A. hydrophila* SS332 was extracted by using a Bacterial Genomic DNA Miniprep kit (Generay, Shanghai, China) following the manufacturer's instructions. Whole-genome sequencing was performed with Illumina (HiSeq 2500, Illumina, United States) and PacBio (PacBio RS II, Pacific Biosciences, United States) platforms. The initial complete genome was assembled from PacBio long reads by Canu v1.7 and then corrected by Pilon with Illumina short reads. Potential open reading frames (ORFs) were predicted using Prodigal and annotated against the UniProt/Swiss-Prot and nonredundant protein databases using the BLASTX program. Annotation of ARGs and mobile genetic elements was performed using online databases, including the Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>) and ISfinder (<https://www-is.biotoul.fr/>). Similar sequences of pSS332-218k showing >50% query coverage were chosen for comparative genomic analysis in the nr/nt database with BLASTN. Amino acid alignment for AFM-1 and representative subclass B1 metallo- β -lactamase (MBLs)

was performed using the program Clustal W (<https://www.genome.jp/tools-bin/clustalw>), and the final output was produced upon processing with the program ESPript 3.0 (<http://esript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>). The maximum likelihood phylogenetic tree of AFM-1 was constructed by MEGA-X software with 1000 bootstrap replications. The molecular weight and pI value of AFM-1 were predicted using ProtParam2. The putative signal peptide cleavage site of *bla*_{AFM-1} was identified by SignalP 5.0. Comparisons of the nucleotide sequences were performed using BLASTN. A plasmid map was generated using GView. Other bioinformatics tools were written using Python and Biopython.

Cloning Experiments

Six ARGs related to antibiotic resistance profiles of *A. hydrophila* SS332 were chosen for cloning, including *bla*_{AFM-1}, *bla*_{OXA-1}, *msr*(E), *mph*(E), *aac*(6)-*Ib10*, and *aph*(3')-*Ia*. The primers of the ARGs with the predicted promoter regions were designed and then synthesized by TSINGKE (Beijing, China) (Table S1). For cloning, *Xba*I (Takara) and *Bam*HI (Takara) restriction endonuclease sites and their protective bases were incorporated into the primers (Table S1, underlined letters). The ARG sequences were PCR amplified from *A. hydrophila* SS332 genomic DNA with the primers. The PCR products were digested

with restriction endonucleases *Xba*I and *Bam*HI and then ligated into an expression vector (pUCP20 or pUCP24) with T4 DNA ligase (Takara). The recombinant plasmids were transformed into *E. coli* DH5 α , which were then grown on Luria-Bertani agar plates supplemented with ampicillin (100 mg/L) or gentamicin (20 mg/L), and then further verified by colony PCR and sequencing. Antimicrobial susceptibility testing for the transformants was performed using the agar dilution method to verify the function of ARGs. Strains DH5 α /pUCP20 and DH5 α /pUCP24 were used as controls. For the AFM-1 inhibition assay, the transformants were incubated with 0.05 mmol/L EDTA.

Results

Antibiotic Resistance Phenotypes and Genome Analysis of *A. hydrophila* SS332

Antimicrobial susceptibility results revealed that of 24 antibiotics tested, *A. hydrophila* SS332 showed high levels of resistance to 15 antibiotics, especially those with MIC levels at or above 1024 μ g/mL, including ampicillin, cefazolin, ceftriaxone, aztreonam, spectinomycin, and roxithromycin. However, SS332 was susceptible to imipenem, meropenem, gentamicin, amikacin, chloramphenicol, florfenicol, levofloxacin, ciprofloxacin and polymyxin B (Table 1).

Table 1 Antibiotic susceptibility profiles of *A. hydrophila* SS332 and transformants

Strain	MIC (μ g/mL)											
	AMP	CFZ	FEP	CTX	FOX	CAZ	CTRX	CSL	CMN	ATM	IMP	MEM
SS332	>1024	>1024	128	512	512	>1024	>1024	512	64	>1024	1	1
ATCC 25922	8	4	0.125	0.06	2	0.25	\leq 0.03	0.06	0.5	0.03	0.25	0.125
<i>E. coli</i> DH5 α	2	1	0.06	0.06	2	0.125	\leq 0.03	0.06	0.25	0.125	0.25	0.125
DH5 α /pUCP24	2	1	0.06	\leq 0.03	2	0.125	\leq 0.03	0.06	0.25	0.03	0.25	0.125
DH5 α /pUCP24- <i>bla</i> _{AFM-1}	16	16	0.125	0.25	16	8	0.25	0.25	0.25	0.03	0.25	0.125
DH5 α /pUCP24- <i>bla</i> _{OXA-1}	64	1	0.06	\leq 0.03	2	1	\leq 0.03	0.06	0.25	0.03	0.25	0.125
	SPT	GEN	KAN	AMK	ERY	ROX	CHL	FFC	NAL	LEV	CIP	PB
SS332	>1024	4	256	8	256	1024	8	2	256	4	\leq 0.25	4
ATCC 25922	8	0.5	4	2	64	64	4	4	8	2	\leq 0.25	\leq 1
<i>E. coli</i> DH5 α	8	0.25	1	1	-	-	-	-	-	-	-	-
DH5 α /pUCP20	8	0.25	1	1	64	256	-	-	-	-	-	-
DH5 α /pUCP20- <i>aac</i> (6)- <i>Ib10</i>	8	0.5	128	16	-	-	-	-	-	-	-	-
DH5 α /pUCP20- <i>aph</i> (3')- <i>Ia</i>	8	0.125	512	2	-	-	-	-	-	-	-	-
DH5 α /pUCP20- <i>msr</i> (E)	-	-	-	-	256	512	-	-	-	-	-	-
DH5 α /pUCP20- <i>mph</i> (E)	-	-	-	-	128	512	-	-	-	-	-	-

Note: -, no detected.

Abbreviations: AMP, ampicillin; CFZ, cefazolin; FEP, cefepime; CTX, cefotaxime; FOX, cefoxitin; CAZ, ceftazidime; CTRX, ceftriaxone; CSL, cefoselis; CMN, cefminox; ATM, aztreonam; IMP, imipenem; MEM, meropenem; SPT, spectinomycin; GEN, gentamicin; KAN, kanamycin; AMK, amikacin; ERY, erythromycin; ROX, roxithromycin; CHL, chloramphenicol; FFC, florfenicol; NAL, nalidixic; LEV, levofloxacin; CIP, ciprofloxacin; PB, polymyxin B.

To better understand the molecular resistance mechanism of SS332, the complete genome sequence of this strain was determined. The genome of SS332 consists of a chromosome (CP071151) and two circular plasmids designated pSS332-218k (CP071152) and pSS332-5k (CP071153). The chromosome comprises 4,792,137 bp with 60.77% G+C content (Table 2). It contains 4474 coding sequences (CDSs), of which 85.09% are protein coding genes with an average ORF length of 911 bp. A total of 122 tRNAs and 10 rRNA operons were predicted. The genome of pSS332-218k is 218,315 bp with 55.30% G+C content, and a total of 243 CDSs were identified with an average length of 749 bp (Table 2).

In addition, a total of 5 known (100% identity) and 59 putative ARGs (>40% identity) were predicted within the whole genome, of which 52 ARGs (such as *bla*_{PER-3}, *mphA*, *bla*_{MOX-7}, *bla*_{OXA-427}, *aadA16*, and *mcr-7.1*) were located on the chromosome (Table S2), 12 ARGs (including *bla*_{AFM-1}, *bla*_{OXA-1}, *msr*(E), *mph*(E), *aac*(6')-Ib10, and *aph*(3')-Ia) were located on the plasmid pSS332-218k (Table S3), and no ARGs were found on plasmid pSS332-5k. Notably, *bla*_{AFM-1}, a *bla*_{NDM}-like gene (804 bp), displayed the highest identity (85%) with the known resistance gene *bla*_{NDM-6}. This gene was first discovered in plasmid pAN70-1 of *Alcaligenes faecalis* strain AN70 (GenBank: MK757441.1) in the NCBI database, but its functional and genetic characterization has not been performed thus far.

ARGs in pSS332-218k Reduced Antimicrobial Susceptibility

To clarify the role of pSS332-218k in the multiple antibiotic resistance phenotypes of SS332, 6 ARGs carried by pSS332-218k were successfully cloned, including *bla*_{AFM-1}, *bla*_{OXA-1}, *aac*(6')-Ib10, *aph*(3')-Ia, *msr*(E) and *mph*(E). The MICs of antibiotics against the recombinant

strains are shown in Table 1. DH5 α /pUCP24-*bla*_{AFM-1} exhibited more than 8-fold higher MICs than DH5 α /pUCP24 for ampicillin (16 μ g/mL), cefazolin (16 μ g/mL), cefoxitin (16 μ g/mL), and ceftazidime (8 μ g/mL), suggesting that AFM-1 was functional in *E. coli* DH5 α . The expression of OXA-1 in *E. coli* DH5 α conferred increased resistance to ampicillin compared to the control strain. The expression of AAC(6')-Ib10 and APH(3')-Ia conferred resistance to kanamycin, with MICs of 128 and 512 μ g/mL, respectively. Meanwhile, *msr*(E) and *mph*(E) conferred resistance to erythromycin and roxithromycin. These transformants exhibited reduced susceptibility to several tested antibiotics, indicating that 6 ARGs on pSS332-218k could be involved in the antibiotic resistance phenotypes of SS332.

Comparative Genomic Analysis of pSS332-218k

To further investigate the molecular characteristics of pSS332-218k, comparative genomic analysis was conducted. Only one sequence with >50% query coverage (75% coverage) with the pSS332-218k sequence was found by the BLASTN program, which is a complete sequence of plasmid pMCR5-045096 (NZ_CP028567) of *A. hydrophila* strain WCHAH045096. WCHAH045096 was isolated from sewage in China in 2015. Comparative genomic analysis showed that pSS332-218k and pMCR5_045096 shared a conserved backbone. However, the *msr*(E), *mph*(E), *bla*_{OXA-1}, and *bla*_{AFM-1} genes were missing in pMCR5_045096 (Figure 1). Comparative genomic analysis suggested that these ARGs might be acquired by horizontal gene transfer.

Phylogenetic Analysis

AFM-1, encoded by *bla*_{AFM-1}, was 267 amino acids in length and displayed the highest level of identity with

Table 2 General features of *Aeromonas hydrophila* SS332 genome

Characteristics	Chromosome	pSS332-218k	pSS332-5k
Size (bp)	4,792,137	218,315	5213
GC content (%)	60.77	55.30	55.02
CDSs	4474	243	6
Hypothetical proteins	1717	176	3
Protein coding (%)	85.09	83.41	73.21
Average ORF length (bp)	911	749	646
tRNAs	122	-	-
rRNAs	10*(5S+16S+23S)1*5S	-	-

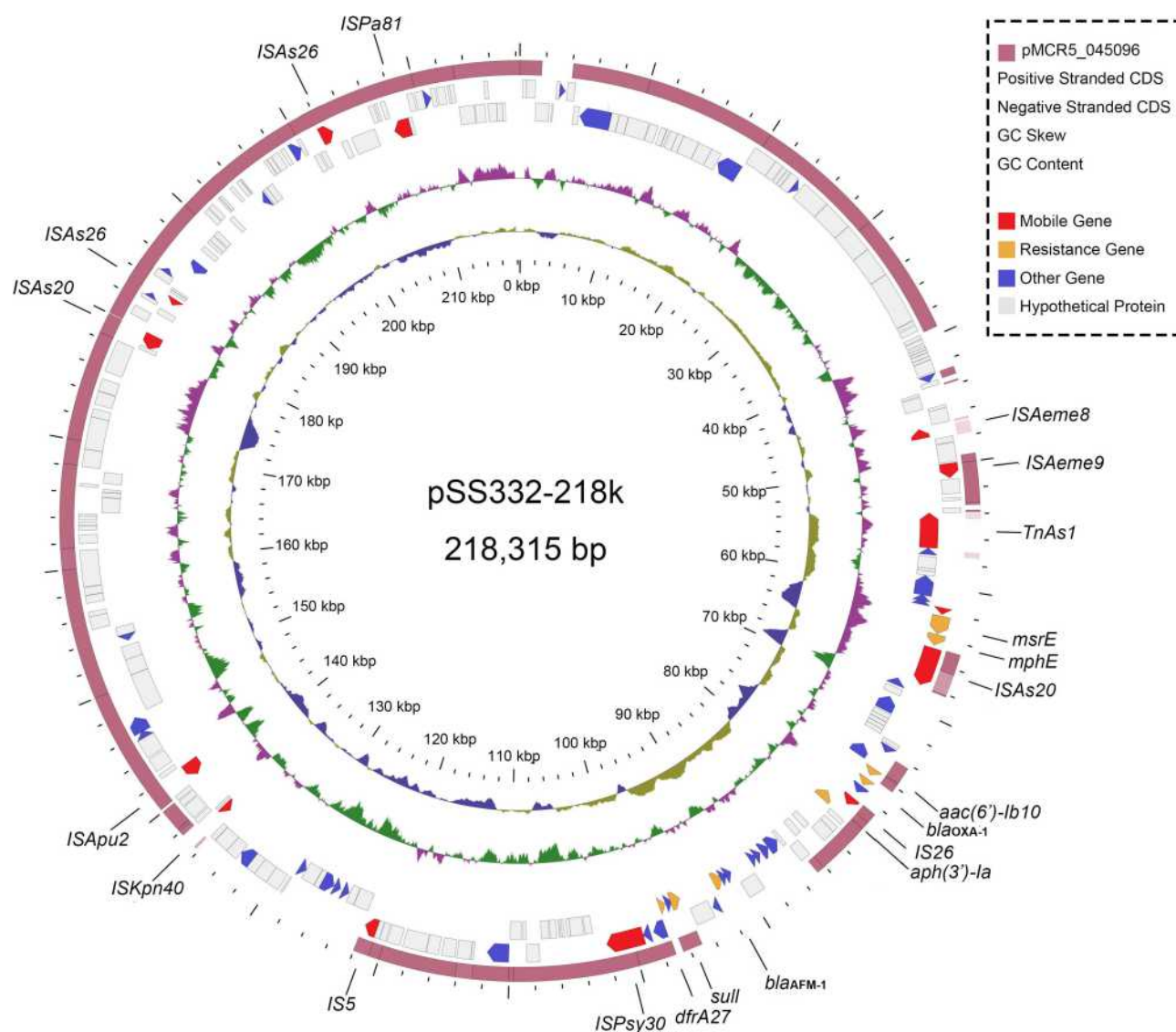


Figure 1 Circular map of complete genome sequences of pSS332-218k and comparison with pMCR5-045096. Circles 1-5 (from inside to outside) represent the information as follows: (1) GC content, (2) GC skew, (3) negative-strand CDSs, (4) positive-strand CDSs and (5) pMCR5-045096. Functional features of pSS332-218k are highlighted in different colors (mobile genetic elements are in red; antimicrobial resistance genes are in yellow; the other known genes are in indigo blue; and hypothetical protein genes are in gray).

NDM β -lactamases (84–86%), followed by EIBla2 (55.79%, ABC63608.1) and FIM-1 (42%, AFV91534.1). A phylogenetic tree was created using non-redundant MBLs of subclass B1. Phylogenetic analysis showed that the closest relatives of AFM-1 were NDM-type enzymes (Figure 2A).

Sequence alignment analysis of AFM-1 and representative MBLs of subclass B1 showed that AFM-1 contains conserved zinc-binding residues, namely, H117, H119, D121, H186, C205 and H247 (black spots, Figure 2B).¹⁵ The inhibition assay indicated that the enzyme activity of AFM-1 was inhibited by EDTA (Figure S1). The requirement for metal ions (usually Zn^{2+}) is the key feature of the

catalytic activity of MBLs.¹⁶ The D87 and D189 residues are essential for the structure and stability of NDM-1 by forming hydrogen bonds around Zn2 and Zn1, respectively (green spots, Figure 2B).¹⁷ A type II signal peptide cleavage site (yellow box, Figure 2B) was predicted between amino acid residues 22 and 23 and yielded a mature protein of 244 amino acids (theoretical mass, 25,991.23 g/mol; predicted pI, 5.7). AFM-1 has seven amino acid substitutions (V85L, A96S, D127G, A135P, S241N, M242T, and V244A) (yellow box, Figure S2) compared with NDM-1 in structurally conserved sequence fragments (black box, Figure S2) according to the sequence alignment of NDM- β -lactamases (Figure S2).¹⁵

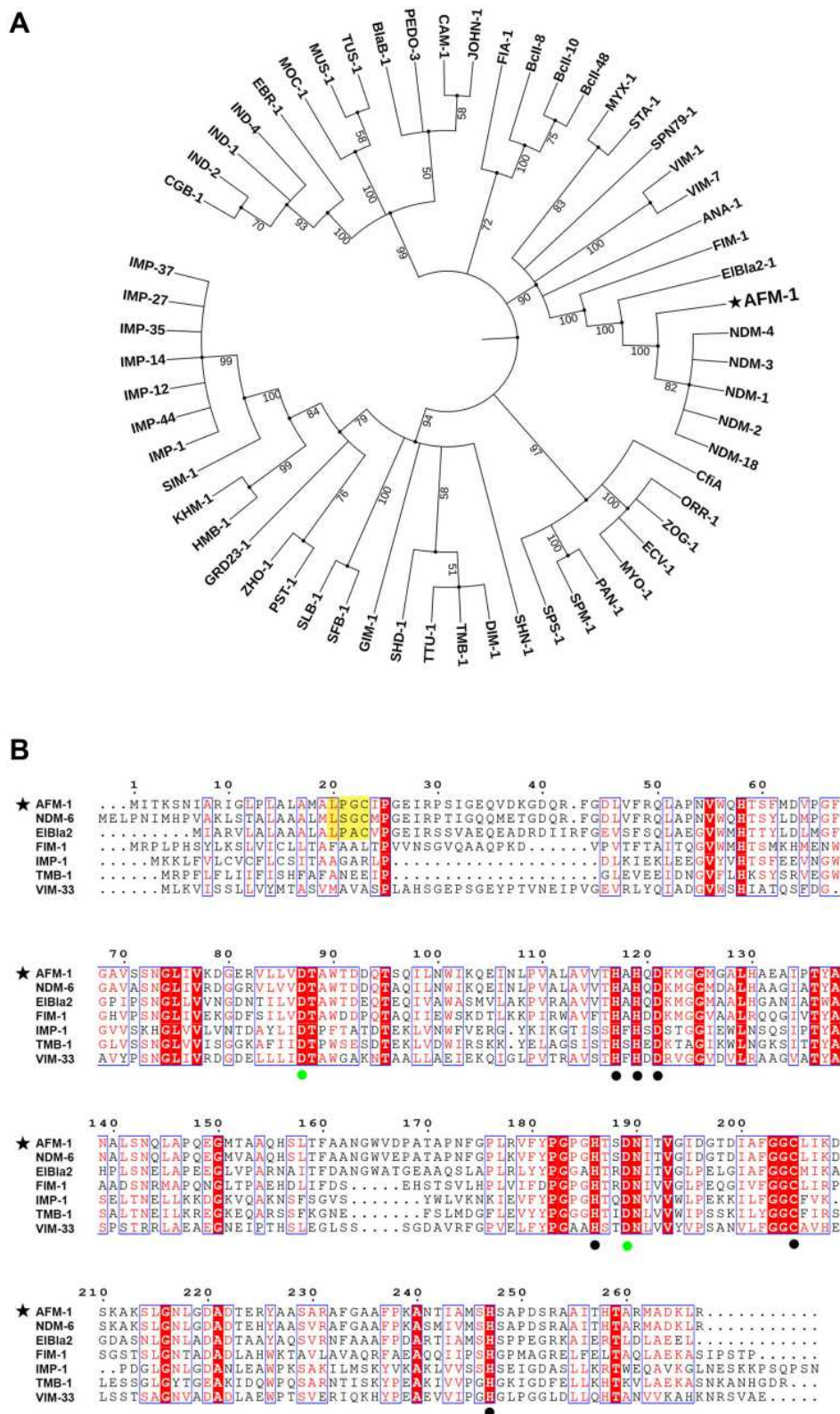


Figure 2 Phylogenetic analysis and sequence alignment of AFM-I. (A) Phylogenetic analysis of the protein sequence using the maximum likelihood method. (B) Sequence alignment analysis of AFM-I and representative MBLs of subclass B1. Residue numbers are positioned above the sequences according to the class B β-lactamase standard numbering scheme; black spots indicate the conserved zinc-binding residues at H117, H119, D121, H186, C205 and H247. Green spots represent the essential residues at D88 and D189 for the stable expression of NDM β-lactamase.

Genetic Context of *bla*_{AFM-1}

Using BLASTN against the GenBank nr database with *bla*_{AFM-1} of SS332 as a query, four sequences identical to *bla*_{AFM-1} were retrieved, including pAN70-1 (MK757441.1) from *Alcaligenes faecalis* strain AN70, plasmid pNFYY023-1 (MT011984.1) from *Comamonas testosteroni* strain NFYY023, *Bordetella trematum* E202 chromosome (CP049957.1) and *Stenotrophomonas maltophilia* NCTC10498 chromosome (CP049956.1). To fully elucidate the genetic context of *bla*_{AFM-1}, comparative genomic analysis was performed on the *bla*_{AFM-1}-flanking regions of approximately 30 kb in the five *bla*_{AFM-1}-carrying sequences, including one from this work (Figure 3A).

The *bla*_{AFM-1} gene in pSS332-218k was located upstream of a Tn3-type transposon carrying the *dfrA5* gene and downstream of an *aph(3')-Ia* gene surrounded by two copies of IS26. A conserved fragment (*bla*_{AFM-1}-*ble*_{MBL}-*trpF*-*ISCR19-2*-*msrB*-*msrA*-*yfcG*-*corA*) was found around *bla*_{AFM-1}. Furthermore, the sequences between *bla*_{AFM-1} and *ISCR19-2* were identical in these five *bla*_{AFM-1}-carrying sequences, but the sequence between *msrB* and *corA* in NCTC10498

shared only 97% identity with that of the other four sequences. *MsrA/B* encode multidrug efflux pumps that play a positive role in the protection of cellular proteins from oxidative stress damage.¹⁸ A similar structure (*msrB*-*msrA*-*ygcG*-*corA*) has also been found around other β -lactamases, such as *bla*_{NDM-1} from *Pseudomonas asiatica*.¹⁹ The conserved fragment containing *bla*_{AFM-1} may improve the adaptability of host bacteria to the environment and might aggravate the spread of this gene among different bacteria.

The *bla*_{AFM-1} gene in pSS332-218k was associated with three novel *ISCR19*-like elements or their truncated genes, *ISCR19-1*, *ISCR19-2* and Δ *ISCR19-3* (Figure 3B). *ISCR19-1* and *ISCR19-2* contain intact transposase genes, but *ISCR19-3* is interrupted at the 5' end (within the transposase gene). The transposases of these three elements were 1542 bp, 1254 bp and 846 bp in length, respectively, and shared 91.0%, 94.0% and 89.1% nucleotide identity to that of *ISCR19*, respectively. The oriS site was identified 245 bp downstream of the stop codon of the transposase gene of *ISCR19-1*, *ISCR19-2* and *ISCR19-3*. These sequences were conserved in *ISCR19*-like elements (Figure 3B). The terIS site was identified 135 bp upstream of the beginning of the

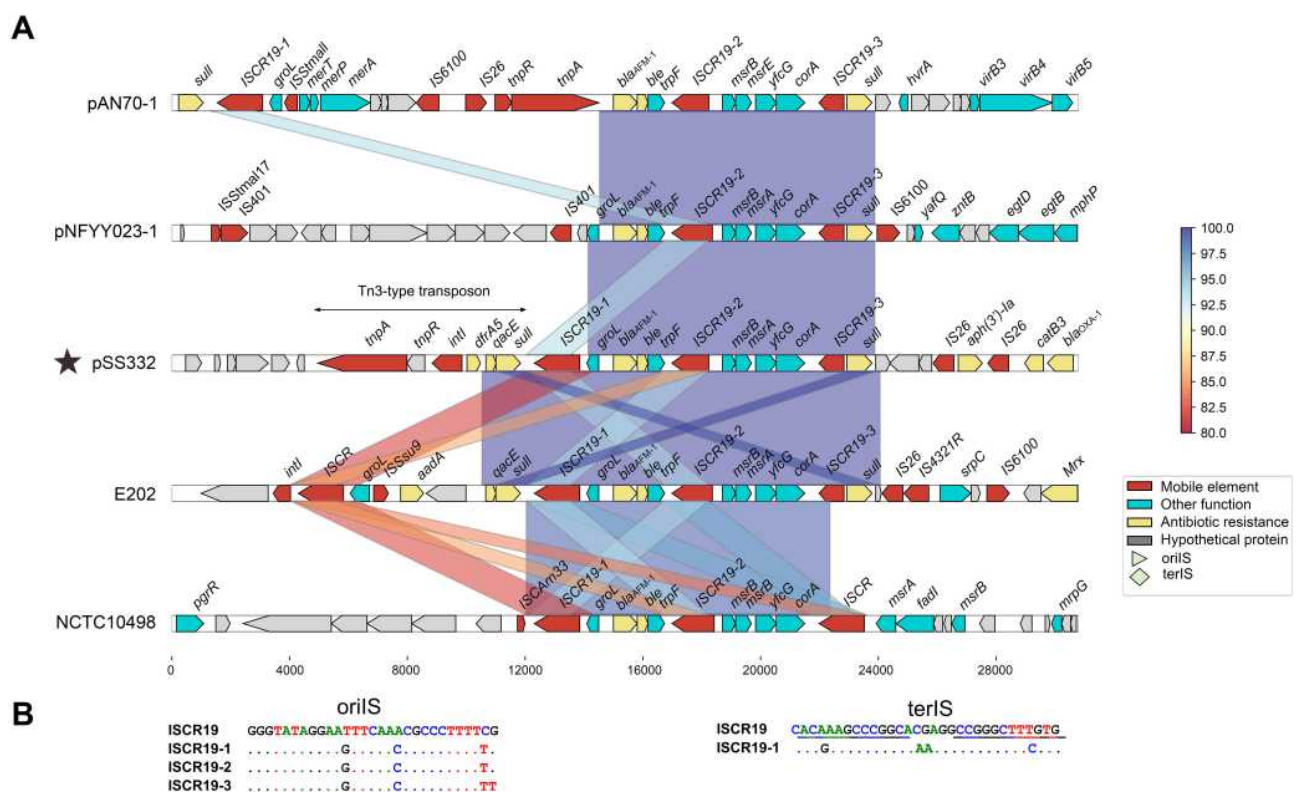


Figure 3 BLAST homology search results. **(A)** The homology of the whole nucleotide sequence of *bla*_{AFM-1} was generated against the nr/nt database using BLASTN. The GenBank accession numbers for the sequences are as follows: *Alcaligenes faecalis* AN70 plasmid pAN70-1, MK757441.1; *Comamonas testosteroni* pNFYY023-1, MT011984.1; *Bordetella trematum* E202, CP049957.1; *Stenotrophomonas maltophilia* NCTC10498, CP049956.1. **(B)** Three novel *ISCR19*-like elements, *ISCR19-1*, *ISCR19-2* and Δ *ISCR19-3*.

transposase gene of *ISCR19-1* but was absent around the corresponding regions of *ISCR19-2* and Δ *ISCR19-3*.

ISCR19-2 was found in all *bla*_{AFM-1}-carrying sequences, but *ISCR19-1* was not identified in pNFYY023-1, and Δ *ISCR19-3* was not found in NCTC10498. Interestingly, *ISCR19-1*, *ISCR19-2* and Δ *ISCR19-3* were associated only with *bla*_{AFM-1} in GenBank. The results suggested that *ISCR19*-like elements were involved in the capture and mobilization of *bla*_{AFM-1}, which was similar to the functions of other *ISCR*-related β -lactamases, such as *bla*_{SPM-1}, *bla*_{AIM-1} and *bla*_{FIM-1}.^{20–22} According to the direction of these *ISCR19*-like elements, we infer that *ISCR19-1* plays a major role in the dissemination of conserved fragments, while *ISCR19-2* was initially involved in the capture of *msrB-msrA-ygcG-corA* regions.

Discussion

In this study, a clinical isolate of *A. hydrophila* was found to be highly resistant to most tested β -lactam, aminoglycoside, and macrolide antibiotics (Table 1). We observed that DH5 α /pUCP24-*bla*_{AFM-1} not only induced resistance to ampicillin, cefazolin, cefoxitin, and ceftazidime but also induced low-level resistance to cefotaxime, ceftriaxone, cefoselis, and cefepime, which are 3rd- and 4th-generation cephalosporins. MICs for these cephalosporins were higher for DH5 α /pUCP24-*bla*_{AFM-1} than for DH5 α /pUCP24, with a more than 8-fold increase for cefotaxime and ceftriaxone, a 4-fold increase for cefoselis, and a 2-fold increase for cefepime (Table 1). In addition, whole-genome sequencing analysis revealed that *A. hydrophila* SS332 possess three chromosomal resistance gene, including extended-spectrum β -lactamases gene *bla*_{PER-3}, AmpC β -lactamases gene *bla*_{MOX-7}, and carbapenem-hydrolyzing oxacillinase gene *bla*_{OXA-427} (Tables S2). MOX-family and PER-family enzymes hydrolyze extended-spectrum cephalosporins.^{23,24} OXA-427 reduced susceptibility to carbapenems and conferred resistance to ceftazidime.²⁵ It might be one reason for the high-level resistance of *A. hydrophila* SS332 to β -lactam antibiotics, including penicillins, monobactams, cephalosporins, and even 3rd- and 4th-generation cephalosporins. However, *bla*_{AFM-1} is unable to hydrolyze imipenem and meropenem. We suspect that *A. hydrophila* SS332 induces low-level resistance to imipenem and meropenem, possibly through chromosomal *bla*_{OXA} gene or efflux pump genes (Table S2). A previous seven-year surveillance study demonstrated that *A. hydrophila* (65.6%, 745/1135) was the most predominant species among clinical *Aeromonas* spp. in

Southwest China from 2011 to 2017.²⁶ And this report also mentioned that *A. hydrophila* has a resistance rates of 30.2% to ceftriaxone; 20% to ceftazidime, cefepime, aztreonam, and ciprofloxacin; and less than 10% to imipenem and meropenem. These findings suggest that the drug resistance of *A. hydrophila* is an increasingly serious problem in clinical infections. However, the role of plasmids in the antibiotic resistance of *A. hydrophila* is poorly investigated. pSS332-218k, a multidrug-resistance plasmid from *A. hydrophila* SS332, carries the antibiotic resistance genes *bla*_{AFM-1}, *bla*_{OXA-1}, *msr*(E), *mph*(E), *aac*(6)-*Ib10*, and *aph*(3')-*Ia*, which conferred resistance to many of the tested antibiotics when expressed in *E. coli*, suggesting that these genes are functional (Table S3). Another multidrug-resistance plasmid, pR148, was also obtained from *A. hydrophila* and had entirely different ARGs, *qacH*, *bla*_{OXA-10}, *aadA1*, *sull*, *tetA*, *tetR*, and *catA2*.²⁷ Plasmids are mobile genetic elements that can transfer ARGs to bacterial chromosomes or gain ARGs through horizontal gene transfer. Therefore, determining ARG transmission may be helpful for decelerating the spread of ARGs.

A conserved genomic DNA fragment (*bla*_{AFM-1}-*ble*_{MBL}-*trpF-ISCR19-2-msrB-msrA-yfcG-corA*) containing *bla*_{AFM-1} was found in all five different AFM-1-producing strains, two of which were located on chromosomes, and the other three of which were located on plasmids (Figure 3A). These results indicated that the *bla*_{AFM-1} gene spread among different strains. Novel *ISCR19*-like elements were found in the region near to AFM-1 (Figure 3B), suggesting that AFM-1 might be transferred from one strain to another, mediated by *ISCR* through rolling-circle transposition.²⁸ The acquisition and spread of ARGs is the predominant factor for the escalation of antibiotic resistance.²⁹ *ISCR* elements are unique mobile elements and can transpose adjacent DNA sequences with a single copy of the element.²⁸ The most worrying feature is that *ISCR* elements are increasingly associated with genes encoding resistance to extended-spectrum β -lactamases, such as *bla*_{PER-3} and *bla*_{OXA-18}.^{30,31}

Since it was first reported in 2009, NDM-1 has become a great public health concern due to its high carbapenem resistance and global dissemination.³² To date, 27 variants of NDM β -lactamase with 1~5 amino acid substitutions have been reported; in NDM-18, an insertion of five amino acids has been found.³³ Among the 28 NDM variants, substitutions of amino acids were identified at 20 different positions. Compared to other NDM variants, AFM-1 has more changes (42 amino acid substitutions) in amino acid

residues and shares a similarity of 86% with NDM-1 (Figure 2 and S1). However, the position that plays the critical role in the enzymatic activity of NDM remains unclear. Further studies, such as site-directed mutagenesis, are needed to clarify this issue. Nevertheless, AFM-1 possesses essential residues class that is indispensable for catalytic activity and a substrate-specific residue class that is an alternative for ampicillin, cefotaxime, or imipenem hydrolysis.¹⁷ (Figure S2). The functions of essential residues and substrate-specific residues for NDM-1 β -lactamase hydrolysis have been well proven As AFM-1 exhibited greatly reduced resistance to all β -lactam antibiotics, it is possible that the substituted residue(s) are responsible for attenuated β -lactam resistance activity of AFM-1 compared with NDM-1.

Conclusion

In this work, the complete genome sequence of multidrug-resistant *A. hydrophila* SS332 was determined. Six ARGs (*bla*_{AFM-1}, *msr*(E), *mph*(E), *aac*(6')-Ib10, and *aph*(3')-Ia) on plasmid pSS332-218k were proven to be functional in *E. coli* DH5 α . Most importantly, a novel *bla*_{NDM}-like gene, *bla*_{AFM-1}, was cloned for the first time and found to confer resistance to ampicillin, ceftazidime, cefoxitin and ceftazidime. However, AFM-1 exhibited a reduced substrate profile and increased MIC value, which may be caused by amino acid substitutions in structurally conserved sequence fragments. Further analysis of the genetic environment of *bla*_{AFM-1} suggested that the risk of spread of *bla*_{AFM-1} among different strains, which was mediated by novel ISCR19-like elements.

Ethics Approval and Consent to Participate

Individual patient data were not involved, and only anonymous clinical residual samples were used in this study. This study followed the principles stated in the Declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest in this work.

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