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Brief Report

Potential KPC-2 carbapenemase reservoir of environmental *Aeromonas hydrophila* and *Aeromonas caviae* isolates from the effluent of an urban wastewater treatment plant in Japan

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Summary

***Aeromonas hydrophila* and *Aeromonas caviae* adapt to saline water environments and are the most predominant *Aeromonas* species isolated from estuaries. Here, we isolated antimicrobial-resistant (AMR) *Aeromonas* strains (*A. hydrophila* GSH8-2 and *A. caviae* GSH8M-1) carrying the carabapenemase bla_{KPC-2} gene from a wastewater treatment plant (WWTP) effluent in Tokyo Bay (Japan) and determined their complete genome sequences. GSH8-2 and GSH8M-1 were classified as newly assigned sequence types ST558 and ST13, suggesting no supportive evidence of clonal dissemination. The strains appear to have acquired bla_{KPC-2}-positive IncP-6-relative plasmids (pGSH8-2 and pGSH8M-1-2) that share a common backbone with plasmids in *Aeromonas* sp. ASNIH3 isolated from hospital wastewater in the United States, *A. hydrophila* WCHAH045096 isolated from sewage in China, other clinical isolates (*Klebsiella*, *Enterobacter* and *Escherichia coli*), and wastewater isolates (*Citrobacter*, *Pseudomonas* and other *Aeromonas* spp.). In addition to bla_{KPC-2}, pGSH8M-1-2 carries an IS*26*-mediated composite transposon including a macrolide resistance gene, *mph*(A). Although *Aeromonas* species are opportunistic pathogens, they could serve as potential environmental reservoir bacteria for carbapenemase and AMR genes. AMR monitoring from WWTP effluents will contribute to the detection of ongoing AMR dissemination in the environment and might provide an early warning of potential dissemination in clinical settings and communities.**

Introduction

Antimicrobial resistance (AMR) is a global issue, and carbapenem-resistant Enterobacteriaceae (CRE) have the potential to facilitate the widespread transmission of AMR. This dissemination is mainly accomplished through mobile genetic elements and the processes of natural competence, transformation and plasmid transconjugation, which can occur in any environment. In addition to clinical settings, AMR genes (ARGs) and AMR bacteria are widely distributed in the environment, particularly in surface waters (Ng *et al.*, 2017), waste water treatment plant (WWTP) effluents (Karkman *et al.*, 2018), soils and animal wastes (Sharma and Reynnells, 2016). In particular, the widespread detection of carbapenemase-producing organisms (CPOs) in the environment is an emerging environmental issue with potentially serious public health implications.

Klebsiella pneumoniae carbapenemase 2 (KPC-2) hydrolyzes all class A β-lactamases, including the most common β-lactams such as carbapenem, which was initially identified from *Salmonella enterica* serovar Cubana (Miriagou *et al.*, 2003). In particular, the widespread detection of CPO is an emerging environmental issue with potentially serious public health implications. Notably, Xu *et al.* (Xu *et al.*, 2018) reported the isolation of KPC-producing *Citrobacter* and *Aeromonas* isolates from sampling sites near a WWTP in China.

Nevertheless, KPC-2-type CPOs have rarely been detected, even in clinical settings, in Japan (Saito *et al.*, 2014), implying that Japan is basically free of KPC-2-type CPO. However, the monitoring of environmental waters in 2017 in Japan revealed a KPC-2-producing *K. pneumoniae* strain, GSU10-3, in an effluent from a WWTP in Tokyo Bay (Japan) (Sekizuka *et al.*, 2018). This finding suggested that the Japanese environment, particularly the water in urban areas, may be contaminated with notable levels of such

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clinically relevant AMR bacteria (Sekizuka *et al.*, 2018), motivating further assessments of the presence of environmental AMR in Japan.

In 2018, we investigated an effluent from a WWTP to identify CPO isolates and isolated *Aeromonas* strains and determined the complete genomes of two isolates carrying the bla_{KPC-2} gene. These genome sequences could suggest the possible role of *Aeromonas* in the environmental dissemination of AMR. A substantial volume of both treated and untreated sewage is continually discharged into urban bays. Thus, our results may point to the role of coastal waters as reservoirs and vectors for AMR bacteria in cities, which can potentially accelerate the spread of ARGs to other non-pathogenic reservoir bacteria. These findings thus raise alarms and highlight the importance for adopting measures to mitigate the spread of AMR in the environment, particularly under the 'One-Health' perspective.

Results and discussion

Isolation of CPE from WWTP effluent

The upper effluent flow of an urban WWTP was collected on 6 August 2018 in the canal (N35.631783, E139.7448151) of Tokyo Bay. CPE was isolated as described previously (Sekizuka *et al.*, 2018). Carbapenemase production by bacterial isolates grown on CHROMagar ESBL plates (CHROMagar, Paris, France) was investigated using the Carba NP test as described previously (Nordmann *et al.*, 2012). KPC-2-producing *K. pneumoniae* GSU10-3 (Sekizuka *et al.*, 2018) and *E. coli* ATCC 25922 were used as a positive and negative control for the assay respectively. Three isolates exhibited a positive reaction in the Carba NP test among 14 representative colonies with differentiated colour indicator, size and form. These isolates were then subjected to whole-genome sequencing (WGS) as described previously (Sekizuka *et al.*, 2018), using an Illumina NextSeq 500 platform with a 300-cycle NextSeq 500 Reagent Kit v2 (2×150-mer). These results confirmed the three clonal isolates of a KPC-2-positive *A. hydrophila* strain (GSH8-2). In addition, four notable colonies were obtained from the same WWTP effluent under 1 μg/ml meropenem (MER) selection, and a strain that reacted positively in the Carba NP test was obtained and sequenced, revealing a KPC-2-positive *A. caviae* strain (GSH8M-1).

In general, *Aeromonas* spp., particularly *A. hydrophila, A. caviae* and *A. veronii*, adapt to saline water environments (Janda and Abbott, 2010) and can therefore be isolated from estuaries. Apart from fish, which are widely reported hosts for Aeromonads, other organisms, including insects, crustaceans, reptiles, birds, and mammals, have also been found to harbour *Aeromonas* species. *Aeromonas* spp. cause opportunistic systemic disease in immunocompromised patients, diarrheal disease and wound infections both in healthy and diseased states (Pearson *et al.*, 2000; Turutoglu *et al.*, 2005; Evangelista-Barreto *et al.*, 2006; Ceylan *et al.*, 2009). Although *Aeromonas* spp. exhibit limited pathogenicity to humans, clinical *A. hydrophila* isolates producing KPC-2 were identified from routine perirectal surveillance culture in hospitalized adult patients in the United States (Hughes *et al.*, 2016). Besides KPC-2, VIM-producing *A. caviae* has been isolated in Israeli hospitals (Adler *et al.*, 2014), GES-24 carbapenemase-producing *A. hydrophila* was isolated from an inpatient in Okinawa, Japan (Uechi *et al.*, 2018), and OXA-181-carbapenemase was found in *A. caviae* (Anandan *et al.*, 2017).

Antimicrobial susceptibility testing of strains GSH8-2 and GSH8M-1 was performed using the Etest (bioMérieux, Marcy-l'Étoile, France) and disk diffusion methods according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines v9.0. Strains GSH8-2 and GSH8M-1 were resistant to the majority of β-lactams except for carbapenems [imipenem (IMI) and MER]. GSH8-2 and GSH8M-1 showed susceptibility and intermediate susceptibility to MER respectively (Table 1). However, the minimum inhibitory concentration (MIC) breakpoints do not always reliably indicate sensitivity during CPE detection because of possible low expression of carbapenemase by bacterial hosts. Therefore, the EUCAST recommends that detection of potential carbapenemase production be carried out with lower screening cut-off values (e.g. MER MIC >0.125 mg/l) (EUCAST, 2017) guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance, indicating that molecular (polymerase chain reaction or WGS) or sensitive enzymatic detection (Carba NP test) are more reliable tests for AMR. Opportunistic pathogens appear to be neglected in AMR studies, and this negative attitude towards microbial testing may increase the risk of AMR transfer through non-pathogenic bacteria behaving as environmental reservoirs (Piotrowska *et al.*, 2017).

Since both strain *A. hydrophila* GSH8-2 and *A. caviae* GSH8M-1 showed positive reactions in the Carba NP test, we performed WGS, which revealed that both strains carry bla_{KPC-2} (Table 2).

WGS analysis of Aeromonas *isolates carrying* bla_{KPC-2}

Summary information regarding the complete chromosome and plasmid sequences for *Aeromonas* isolates GSH8-2 (Fig. 1) and GSH8M-1 (Fig. 2) are shown in Table 2. Multilocus sequence typing (MLST) based on the genomic data assigned both strains to novel sequence types (ST), classifying GSH8-2 and GSH8M-1 as ST558 and ST13 respectively (Table 2).

To trace the potential clonal sources of the *Aeromonas* GSH8-2 and GSH8M-1 strains, we performed core-genome phylogenetic analysis using 108 and 30 publicly available *A. hydrophila* and *A. caviae* genome sequences (at 29 October

Table 1. Antimicrobial susceptibility test (MIC, mg L^{-1}).

Antimicrobial agents	*A. hydrophila* GSH8-2		*A. caviae* GSH8M-1		Electrotransformant in *E. coli* DH10B $T1^R$					
					-		pGSH8-2		pGSH8M-1-2	
AMS	128	R	128	R	1.5	S	128	R	48	R
CEP	4	I	16	R	0.032	S	0.75	S	0.75	S
CTA	32	R	>32	R	0.032	S	1.5	I	0.75	I
CTZ	2	I	16	R	0.032	S	2	I	1.5	I
IMI	1	S	4	I	0.19	S	1	S	0.75	S
MER	1	S	4	I	≤0.125	S	0.19	S	0.125	S
AZT	8	R	32	R	0.064	S	12	R	12	R
AMI	2	S	16	I	1	S	1.5	S	1.5	S
GEN	0.5	S	1	S	0.38	S	0.38	S	0.5	S
TOB	2	S	8	R	0.25	S	0.25	S	0.38	S
TET	8	-	2	-	0.75	-	1	-	1	-
CIP	0.5	I	1	R	≤0.002	S	≤0.002	S	≤0.002	S
LEV	2	R	2	R	0.004	S	0.004	S	0.004	S
AZI	64	-	32	-	2	-	3	-	96	-
TRS	0.25	S	1	S	0.016	S	0.023	S	0.023	S
CHL	1	S	2	S	1.5	S	1.5	S	1.5	S
COL	2	S	0.5	S	0.032	S	0.047	S	0.047	S

AMS, ampicillin-sulbactam; CEP, cefepime; CTA, cefotaxime; CTZ, ceftazidime; IMI, imipenem; MER, meropenem; AZT, aztreonam; AMI, amikacin; GEN, gentamicin; TOB, tobramycin; TET, tetracycline; CIP, ciprofloxacin; LEV, levofloxacin; AZI, azithromycin; TRS, trimethoprim-sulfamethoxazole; CHL, chloramphenicol; COL, colistin.
-, not defined in EUCAST.

2018), including the respective draft genomes with at least 40× read coverage (strain phylogenies are presented in the Supporting Information Figs. S1 and S2). Those retrieved sequences were compared using bwaMEM read mapping against either the GSH8-2 or GSH8M-1 complete genome sequences as reference respectively. After excluding repeat regions from the whole genome sequences, single nucleotide variants (SNVs) were identified in the core genomes of GSH8-2 and GSH8M-1 (see Supporting Information Figs. S1 and S2 respectively). Core-genome MLST (cgMLST) was then performed using these SNVs, and the phylogenies were generated using the maximum-likelihood phylogenetic method with FastTree v2.1.10.

The core genome of the *A. hydrophila* strains constituted 48.95% of the total genome based on 491 764 SNVs (Supporting Information Fig. S1). The phylogeny placed GSH8-2 in one of multiple clusters, unrelated to previously identified clinical and environmental isolates (Supporting Information Fig. S1). No pathogenic fish clonal cluster was observed among isolates from the United States or China. To date, *A. hydrophila* has mostly been isolated as a fish pathogen, and from environmental sources such as rivers and sewage treatment plants, as well as from clinical specimens from patients treated with leech therapy. Thus far, only two strains, including GSH8-2 in this study, have been recovered from sewage treatment plants, making GSH8-2 useful for characterizing the genetic and phenotypic features of inhabitants of waste processing or decontamination sites.

The core genome of the *A. caviae* strains constituted 70.53% of the whole genome, and the phylogenetic analysis used 229 493 SNVs (Supporting Information Fig. S2). The core-genome phylogeny of *A. caviae* strains was variable,

Table 2. Whole genome information of *Aeromonas* spp. from the WWTP effluent.

Organism/Strain	Replicon	GC%	Length (bp)	MLST[a]	Inc type	Drug Resistance Gene	GenBank ID	BioProject ID	BioSample ID
Aeromonas hydrophila GSH8-2									
	Chromosome	61.07	4 962 478	ST558	-	*cepS*, *cphA2*, $bla_{OXA-726}$ *mph*(A), *tet*(E)	AP019193	PRJDB6962	SAMD00144877
	pGSH8-2	58.20	39 071	-	IncP-6	bla_{KPC-2}	AP019194	PRJDB6962	SAMD00144877
Aeromonas caviae GSH8M-1									
	Chromosome	61.00	4 611 279	ST13	-	bla_{MOX-12}, $bla_{OXA-780}$, *mcr-3.18*, *mcr-3* variant	AP019195	PRJDB6962	SAMD00144878
	pGSH8M-1-1	54.22	153 814	-	UT	$bla_{OXA-669}$, *aac(6′)-Ia*, *aadA2*, 3x *sul1*	AP019196	PRJDB6962	SAMD00144878
	pGSH8M-1-2	58.76	53 629	-	IncP-6	bla_{KPC-2}, *mph*(A)	AP019197	PRJDB6962	SAMD00144878
	pGSH8M-1-3	52.25	8,098	-	UT	-	AP019198	PRJDB6962	SAMD00144878

-, not available; UT, unknown type.
The short- and long-read sequences for DNA-Seq were deposited in the DNA Data Bank of Japan (BioProject PRJDB6962; BioSample SAMD00144877 for GSH8-2 and SAMD00144878 for GSH8M-1).
pubMLST for *Aeromonas* spp. (https://pubmlst.org/aeromonas/).

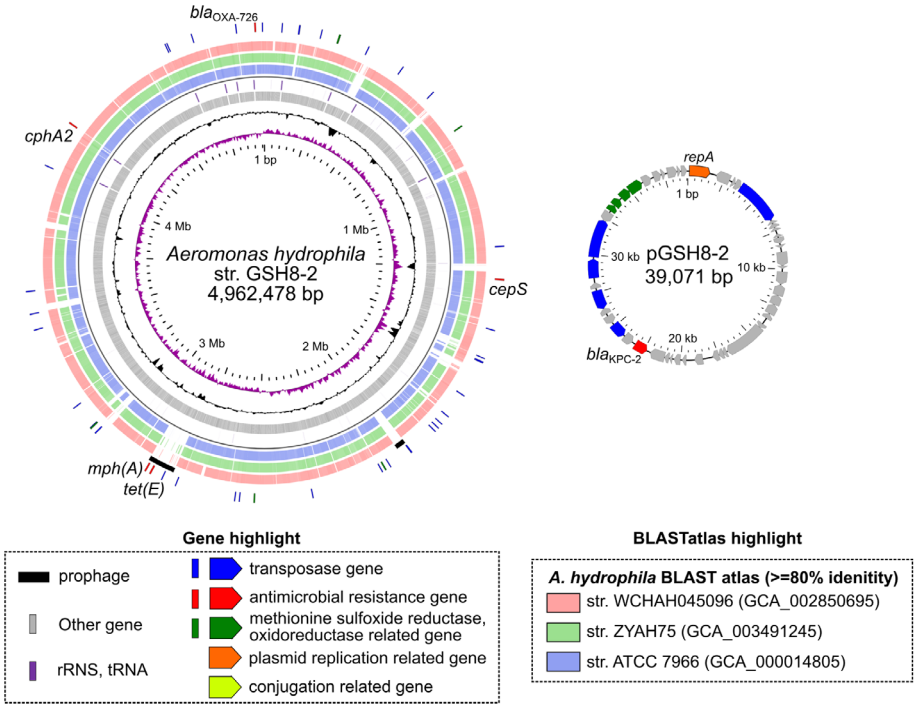


Fig. 1. Schematic circular representation of complete genome sequences of KPC-2-producing *A. hydrophila* GSH8-2. Short paired-end whole-genome sequencing was performed using an Illumina NextSeq 500 platform with a 300-cycle NextSeq 500 Reagent Kit v2 (2 × 150-mer). The complete genome sequences of the strains were determined using a PacBio Sequel sequencer for long-read sequencing [Sequel SMRT Cell 1 M v2 (4/tray); Sequel sequencing kit v2.1; insert size, approximately 10 kb). De novo assembly was performed using Canu version 1.4 (Koren *et al.*, 2017), minimap version 0.2-r124 (Li, 2016), racon version 1.1.0 (Vaser *et al.*, 2017) and circulator version 1.5.3 (Hunt *et al.*, 2015). Error correction of tentative complete circular sequences was performed using Pilon version 1.18 with Illumina short reads (Walker *et al.*, 2014). Annotation was performed in Prokka version 1.11 (Seemann, 2014), InterPro v49.0 (Finn *et al.*, 2017) and NCBI-BLASTP/BLASTX. Circular representations of complete genomic sequences were visualized using GView server (Petkau *et al.*, 2010). AMR genes were identified by homology searching against the ResFinder database (Zankari *et al.*, 2012). The class 1 integron was assigned in the INTEGRALL database (http://integrall.bio.ua.pt/) (Moura *et al.*, 2009). Visualization of comparative plasmid ORFs organization was performed using Easyfig (Sullivan *et al.*, 2011). For representation of chromosomal DNA, from the inside: slot 1, GC skew; slot 2, GC content; slot 3, ORFs; slot 4, rRNA/tRNA; slots 5–7, BLASTatlas conserved gene analysis indicating three relative strains (see also Supporting Information Fig. S1); slot 8, prophage; slot 9, notable ARGs or ARG-related genes (transposase, ARGs and reductases). In representations of circular plasmids, notable ORFs are highlighted as the indicated colour.

and no relevant clinical or environmental isolates related to GSH8M-1 were found (Supporting Information Fig. S2). To date, too few *A. caviae* genomes have been sequenced to determine the original source or clonal dissemination. In contrast to pathogenic *A. hydrophila* strains, *A. caviae* has been more frequently isolated from environmental sources such as sewage treatment plants than from clinical settings. Similar to GSH8-2, GSH8M-1 may be used for characterizing the genetic and phenotypic features of waste processing or decontamination site inhabitants.

ARGs in plasmids and chromosomes

Aeromonas hydrophila GSH8-2. Aeromonas hydrophila GSH8-2 harbours a 39.1-kb IncP-6 replicon plasmid, pGSH8-2, carrying bla_{KPC-2} (Table 2 and Fig. 1). In addition, the strain was found to carry multiple β-lactamase genes [*cepS* (FOX/MOX family class C β-lactamase, 95% identity), *cphA2* (CphA family subclass B2 metallo-β-lactamase, 98% identity), $bla_{OXA-726}$ (class D β-lactamase)] on its chromosome. These are intrinsic ARGs in *A. hydrophila* strains. Intriguingly, it also carried chromosomal copies of macrolide 2′-phosphotransferase [*mph*(A)] and tetracycline efflux MFS transporter [*tet*(E)], which were found within a potential prophage (2805–2905 kb, shown in Fig. 1). BLASTatlas analysis suggested that these horizontally acquired phage elements are GSH8-2 strain-specific, and are similar to elements from the ATCC 7966 type strain WCHAH045096 (isolated from sewage in China in 2015, bla_{KPC-2} positive) and ZYAH75 (isolated from a human wound in China in 2015) (slots 5–7 in Fig. 1). The detection of *mph*(A) and *tet*(E) on a prophage in the chromosome of GSH8-2 could contribute to the reduced susceptibility to azithromycin and tetracycline respectively (Table 1).

Aeromonas caviae GSH8M-1. Aeromona caviae GSH8M-1 also harbours the IncP-6 replicon plasmid pGSH8M-1-2 (53.6 kb), which carries bla_{KPC-2}, as well as additional acquired elements, including *mph*(A), on the IS*26* composite transposon (Table 2 and Fig. 2). pGSH8M-1-1 (153.8 kb) is an unknown-type replicon plasmid, but it shares a partial similar region (47%–51%) with *Aeromonas salmonicida* plasmids (pS44-1, pAsa4c, pAsa4b and plasmid 4) (data not shown). pGSH8M-1-1 carries an antimicrobial

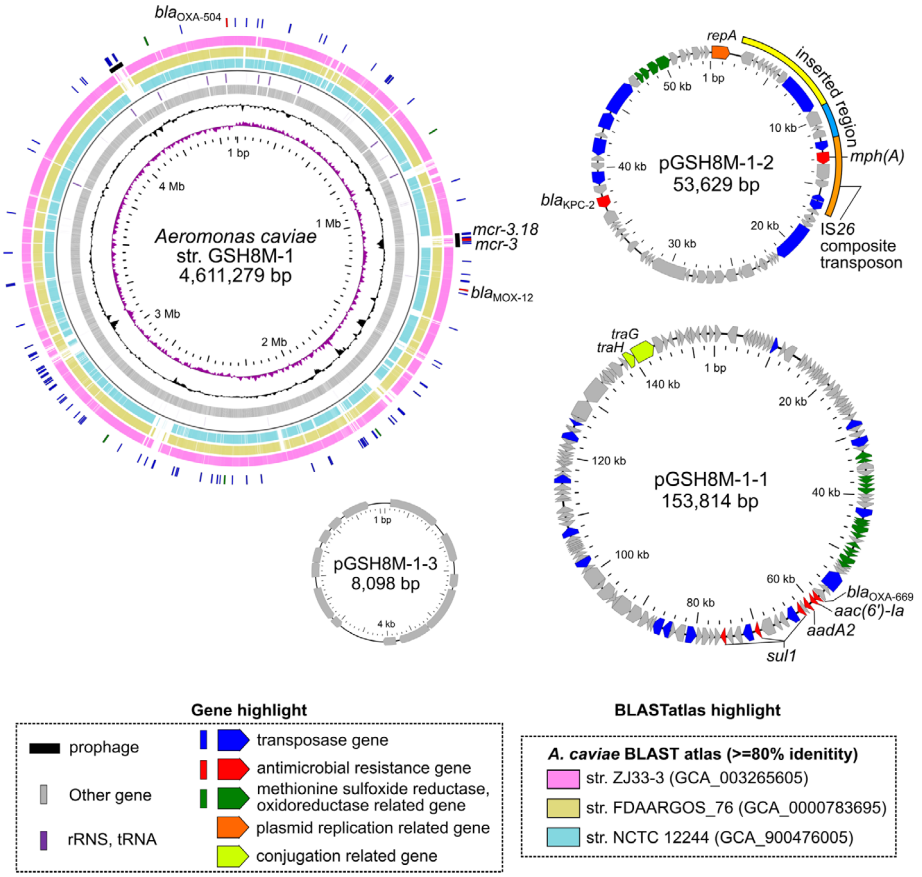
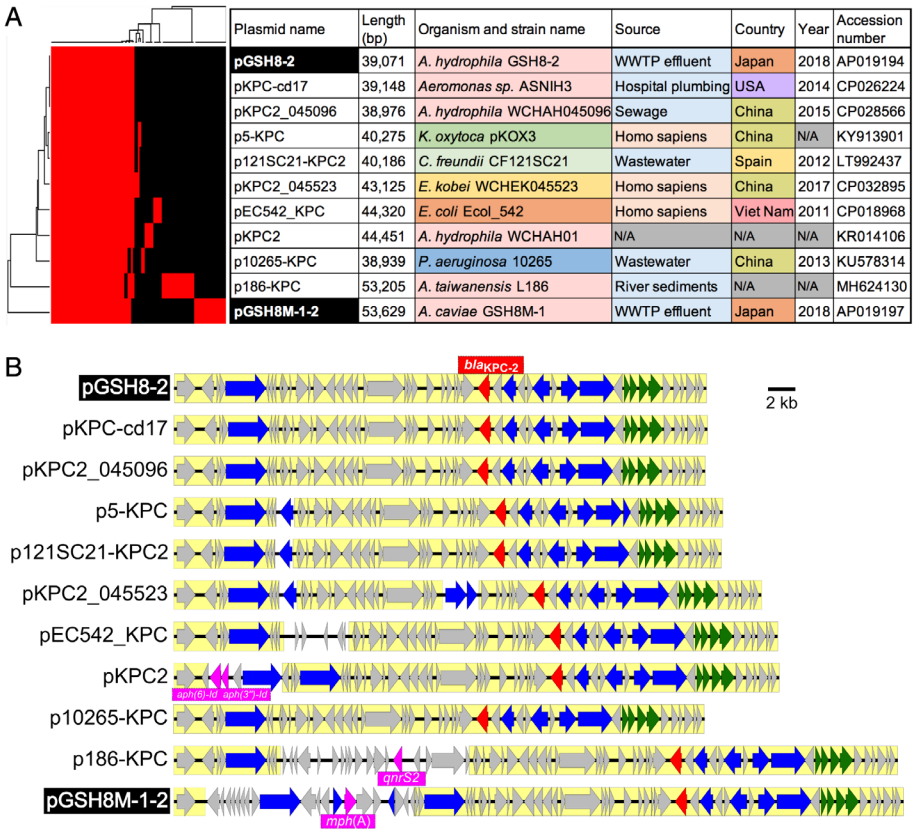


Fig. 2. Schematic circular representation of complete genome sequences of KPC-2-producing *A. caviae* GSH8M-1. See the legend to Fig. 1 for methods and descriptions of representations. Slots 5–7, BLASTatlas conserved gene analysis indicates three relative strains (see also Supporting Information Fig. S2). Marked acquired elements in pGSH8M-1-2 compared to pGSH8-2 are highlighted in the outer slot.

resistance island (3.9–28.0 kb), which includes a class I integron, assigned as In1665 [$bla_{OXA-669}$, *aac(6′)-Ia*, *aadA2*, *qacEdelta*, *sul1*], and two additional sulfonamide-resistant dihydropteroate synthase (*sul1*) genes. pGSH8M-1-3 (8.0 kb) is a small and unknown-type replicon plasmid, sharing a partial similarity region (<25%) with *Aeromonas* spp. plasmids and does not carry any ARGs. Multiple ARGs intrinsic to *A. caviae* strains [bla_{MOX-12} (CMY-1/MOX family class C β-lactamase], as well as $bla_{OXA-780}$ (class D β-lactamase) were identified on the strain's chromosome. Two *mcr-3* variants (*mcr-3.18* and an *mcr-3* variant) are located on a potential prophage in GSH8M-1 (1112–1155 kb, shown in Fig. 2). BLASTatlas analysis suggested that this horizontally acquired phage similar to GSH8M-1 strain-specific elements is comparable to sequences from the NCTC 12244 type strain ZJ33-3 (from a human rectal swab in China in 2017, *mcr-3.3*-positive) and FDAARGOS_76 (from a human stool in the United States in 2013) (slots 5–7 in Fig. 2). Detection of *mcr3* gene variants on a prophage in the chromosome of GSH8M-1 is of note despite its susceptibility to colistin (Table 1). Indeed, *mcr-3*-positive *Aeromonas* spp. have been reported and widely isolated from humans, retail meat and environmental water (Shen *et al.*, 2018). Most of these *mcr-3*-positive strains showed moderate susceptibility to colistin and failed in transconjugation of *mcr-3* gene variants to *E. coli* recipients (Shen *et al.*, 2018), consistent with our findings that *mcr-3* variants are located on a prophage. Thus, *Aeromonas* spp. and the aquatic environment might be a potential source of *mcr* genes for exaptation to a hypertonic marine environment (Cabello and Godfrey, 2018).

Structural comparison of KPC-2 plasmids

Both *Aeromonas* strain GSH8-2 and GSH8M-1 carry a bla_{KPC-2}-positive plasmid (pGSH8-2 and pGSH8M-1-2 respectively) and share a 39–40-kb backbone of IncP-6 replicon plasmids (Fig. 3). pGSH8-2 shares a common backbone with plasmids in isolates from environmental sources. These include plasmid pKPC-cd17 in *Aeromonas* sp. ASNIH3 isolated from hospital wastewater in Bethesda (USA), in 2014, and plasmid pKPC2_045096 in *A. hydrophila* subsp. *hydrophila* strain WCHAH045096 isolated from sewage in Sichuan (China) in 2015 (Fig. 3A). pGSH8-2-related IncP-6 plasmids might be fundamental KPC-2-positive plasmids in *A. hydrophila*, which potentially serves as an environmental reservoir for KPC-2 carbapenemase. Similar IncP-6 plasmids found in *Klebsiella*, *Citrobacter*, *Enterobacter*, *E. coli*, *Pseudomonas* and other *Aeromonas* spp., have acquired additional ARG elements (*aph(6)-Id* and *aph(3″)-Id* in



Plasmid name	Length (bp)	Organism and strain name	Source	Country	Year	Accession number
pGSH8-2	39,071	*A. hydrophila* GSH8-2	WWTP effluent	Japan	2018	AP019194
pKPC-cd17	39,148	*Aeromonas sp.* ASNIH3	Hospital plumbing	USA	2014	CP026224
pKPC2_045096	38,976	*A. hydrophila* WCHAH045096	Sewage	China	2015	CP028566
p5-KPC	40,275	*K. oxytoca* pKOX3	Homo sapiens	China	N/A	KY913901
p121SC21-KPC2	40,186	*C. freundii* CF121SC21	Wastewater	Spain	2012	LT992437
pKPC2_045523	43,125	*E. kobei* WCHEK045523	Homo sapiens	China	2017	CP032895
pEC542_KPC	44,320	*E. coli* Ecol_542	Homo sapiens	Viet Nam	2011	CP018968
pKPC2	44,451	*A. hydrophila* WCHAH01	N/A	N/A	N/A	KR014106
p10265-KPC	38,939	*P. aeruginosa* 10265	Wastewater	China	2013	KU578314
p186-KPC	53,205	*A. taiwanensis* L186	River sediments	N/A	N/A	MH624130
pGSH8M-1-2	53,629	*A. caviae* GSH8M-1	WWTP effluent	Japan	2018	AP019197

Fig. 3. Structural comparison of the bla_{KPC-2}-positive plasmids**.** A. Heat map representation of orthologous gene clustering of bla_{KPC-2}-positive IncP-6 plasmids. B. Comparison of gene structure among plasmids shown in panel (A). Genes for bla_{KPC-2}, ARGs, transposase, methionine sulfoxide reductase/oxidoreductase, and others are highlighted with red, magenta, blue, green, and grey, respectively, as shown in Fig. 1.

pKPC2; *qnrS2* in p186-KPC; *mph*(A) in pGSH8M-1-2) (Fig. 3B). Sequence alignments suggested that IncP-6 plasmids [equivalent to IncG/U (Haines *et al.*, 2006)] are broadly disseminated in multiple organisms (Fig. 3A), strongly indicating that IncP-6 replicon shows ability of replication over a broad host range, as previously described (Haines *et al.*, 2005).

Within this bla_{KPC-2}-positive IncP-6 plasmid family, less pathogenic *Aeromonas* (Wang *et al.*, 2017)*, Citrobacter* (Yao *et al.*, 2017) and *Pseudomonas* (Dai *et al.*, 2016) were isolated mostly from environments such as waste water, hospital plumbing (Weingarten *et al.*, 2018), and river sediments (Dai *et al.*, 2016), whereas bacteria with higher pathogenicity, such as *Klebsiella oxytoca* (Wang *et al.*, 2017)*, Enterobacter kobei* and *E. coli*, were isolated from clinical specimens, indicating that the isolation source may depend on the pathogenicity of the bacterial host (Fig. 3). In particular, IncP-6 plasmids [described as IncU in Piotrowska *et al.* (Piotrowska and Popowska, 2015)] have been characterized as multiple AMR plasmids. In line with the present study, a previous study demonstrated that the IncP-6 (IncU) replicon plasmid also contributes to the dissemination of the tetracycline resistance gene *tetA* between *Aeromonas* species and *E. coli* isolates from fish farm environments, hospital sewage and a hospital patient (Rhodes *et al.*, 2000)

When compared with pGSH8-2, pGSH8M-1-2 was found to carry an additional elements, the first of which is a Tn*3* family transposon (inverted repeats at nt 27 425– 34 564: GGGGATGTAAGCCGGAACCCCAGAAATTTCCGTCAC-CCTAG –CTGGGCTGACGGAAATTTCTGGGATTCCGG CGTACAACCCC), and the second is the IS*26*-mediated composite transposon carrying *mph*(A) family macrolide 2′-phosphotransferase (Fig. 1B). A Blastp homology search indicated that this Mph(A) shares 100% identity with those found in 39 *E. coli*, 14 *Klebsiella pneumoniae*, 1 *Salmonella* Typhimurium and 1 *Shigella flexneri* strains. In addition, IS*26* is a ubiquitous IS in Enterobacteriaceae, indicating that *mph*(A) acquisition could take place through IS*26*-mediated recombination with other potential *mph*(A) resources.

KPC-2 plasmids reduced antimicrobial susceptibility

To characterize the single effect of each KPC-2-positive plasmid (pGSH8-2 or pGSH8M-1-2), electro-transformants carrying the KPC-2-plasmid under an *E. coli* DH10B $T1^R$

strain background were obtained (see detailed experimental methods in the Supporting Information), and the MIC values were determined (Table 1). These electro-transformants exhibited significantly reduced susceptibility to all tested β-lactams but not to other antimicrobials except for azithromycin. pGSH8M-1-2 carries *mph*(A) (Fig. 2), which could be involved in the macrolide resistance in the GSH8M-1 strain (reduced susceptibility to azithromycin from 2 to 96 mg/l).

Multiple sulfo-related reductase enzymes

Intriguingly, *A. caviae* GSH8M-1 has acquired a genomic island including multiple sulfo-related reductase enzymes such as glutathione S-transferase (GST), two peptide methionine sulfoxide reductases (MsrA/MsrB), two glutaredoxins, two zinc-type alcohol dehydrogenase-like proteins, two 3-ketoacyl-CoA thiolases and other oxidoreductases (green highlighted ORFs in Fig. 2). In total, four sets of redundant MsrA/B were identified in *A. caviae* GSH8M-1 (one on its chromosome, two on pGSH8M-1-1 and one on pGSH8M-1-2). Likewise, *A. hydrophila* GSH8-2 plasmid pGSH8M-1-2 also carries GST and MsrA/B reductase (Fig. 1). Such multiple sulfo-related reductase enzymes might contribute to adaptation to oxidative stresses encountered in WWTP environments or during antiseptic treatment.

Conclusions and prospects

We isolated a potential KPC-2 carbapenemase reservoir in environmental *Aeromonas* isolates from a WWTP effluent in Tokyo Bay. KPC-type CPOs have rarely been detected, even in clinical settings, in Japan. Thus, although *Aeromonas* species are opportunistic pathogens, such rather neglected pathogens might play a key role as environmental antimicrobial resistance reservoirs.

In conclusion, AMR monitoring of WWTP effluent contributes to the detection of ongoing bacterial AMR dissemination in the environment, and can provide early warning of the potential dissemination in clinical settings and communities. CPO-colonized and infected patients may disseminate AMR bacteria in excreta, which encounter active broad-spectrum antimicrobials in hospital sewage systems, leading to the perfect selection pressure for the exchange of resistance genes between clinical pathogens and environmental bacteria (Taylor *et al.*, 2011; Picao *et al.*, 2013). Indeed, KPC-type CPOs have been found in hospital effluents in Brazil, China, and the United States (Chagas *et al.*, 2011; Zhang *et al.*, 2012; Picao *et al.*, 2013; Woodford *et al.*, 2014; Weingarten *et al.*, 2018). Subsequently, identification of the presence of CPO in polluted aquatic environments such as recreational waters indicated a potential threat to beach frequenters (de Araujo *et al.*, 2016; Paschoal *et al.*, 2017; Leonard *et al.*, 2018).

Because contamination in the environment can serve as AMR reservoirs, potentially causing infectious diseases or infecting healthy carriers through consumption of contaminated water or food, or through recreational activities (Schijven *et al.*, 2015; Leonard *et al.*, 2018), a comprehensive approach will be required to uncover environmental contamination through WWTP effluents, and more attention should be focused on non- or opportunistic pathogens as environmental AMR reservoirs.

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Authors' contributions

TS and MK contributed to the isolation of the KPC-2-positive *Aeromonas* strains. TS and MH performed antimicrobial susceptibility testing. YI performed the genome sequencing. TS and KY performed the comparative genome analysis. MK wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supplementary Information

Supplemental Figure S1 Core genome phylogeny of *Aeromonas hydrophila* GSH8-2 among publicly available 108 strains (updated at 2018/10/29).

All publicly available draft genome sequences of *Aeromonas* strains were retrieved. Analysis of conserved plasmid gene sequences was performed with BLASTn searches (≥99% nt identity), followed by visualization using FriPan (http://drpowell.github.io/FriPan/). Using the complete genome sequence of GSH8-2 (4,962,478 bp), bwa-read mapping analysis identified 48.95% of the full genome as core-genome sequence, and a total of 491,764 single nucleotide variations (SNVs) were identified, excluding prophages and repeated regions.

Supplemental Figure S2 Core genome phylogeny of *Aeromonas caviae* GSH8M-1 among publicly available 30 strains (updated at 2018/10/29).

Using the complete genome sequence of GSH8M-1 (4,611,279 bp), bwa-read mapping analysis identified 70.53% of the full genome as core-genome sequence, and a total of 229,493 single nucleotide variations (SNVs) were identified, excluding prophages and repeated regions.