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ENVIRONMENTAL MICROBIOLOGY

Genomic Analysis of Carbapenem-Resistant *Comamonas* in Water Matrices: Implications for Public Health and Wastewater Treatments

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ABSTRACT *Comamonas* spp. are Gram-negative bacteria that catabolize a wide range of organic and inorganic substrates. *Comamonas* spp. are abundant in aquatic and soil environments, including wastewater, and can cause opportunistic infections in humans. Because of their potential in wastewater bioaugmentation and bioremediation strategies, the identification of *Comamonas* species harboring genes encoding carbapenemases and other clinically important antibiotic resistance genes warrant further investigation. Here, we present an analysis of 39 whole-genome sequences comprising three *Comamonas* species from aquatic environments in South Australia that were recovered on media supplemented with carbapenems. The analysis includes a detailed description of 33 *Comamonas denitrificans* isolates, some of which carried chromosomally acquired bla_{GES-5}, bla_{OXA}, and aminoglycoside resistance (*aadA*) genes located on putative genomic islands (GIs). All bla_{GES-5}- and bla_{OXA}-containing GIs appear to be unique to this Australian collection of *C. denitrificans*. Notably, most open reading frames (ORFs) within the GIs, including all antimicrobial resistance (AMR) genes, had adjacent *attC* sites, indicating that these ORFs are mobile gene cassettes. One *C. denitrificans* isolate carried an IncP-1 plasmid with genes involved in xenobiotic degradation and response to oxidative stress. Our assessment of the sequences highlights the very distant nature of *C. denitrificans* to the other *Comamonas* species and its apparent disposition to acquire antimicrobial resistance genes on putative genomic islands.

IMPORTANCE Antimicrobial resistance (AMR) poses a global public health threat, and the increase in resistance to "last-resort drugs," such as carbapenems, is alarming. Wastewater has been flagged as a hot spot for AMR evolution. *Comamonas* spp. are among the most common bacteria in wastewater and play a role in its bioaugmentation. While the ability of *Comamonas* species to catabolize a wide range of organic and inorganic substrates is well documented, some species are also opportunistic pathogens. However, data regarding AMR in *Comamonas* spp. are limited. Here, through the genomic analyses of 39 carbapenem-resistant *Comamonas* isolates, we make several key observations, including the identification of a subset of *C. denitrificans* isolates that harbored genomic islands encoding carbapenemase bla_{GES-5} or extended-spectrum β-lactamase bla_{OXA} alleles. Given the importance of *Coma-monas* species in potential wastewater bioaugmentation and bioremediation strategies, as well as their status as emerging pathogens, the acquisition of critically important antibiotic resistance genes on genomic islands warrants future monitoring.

Editor Hideaki Nojiri, University of Tokyo

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The authors declare no conflict of interest.

Received 13 April 2022
Accepted 25 May 2022
Published 16 June 2022

KEYWORDS *Comamonas denitrificans*, wastewater, whole-genome sequencing (WGS), genomic islands, carbapenemase, incP-1, antimicrobial resistance (AMR), *Comamonas*, bla_{GES-5}

Comamonas spp. are Gram-negative bacilli that reside within the family *Comamonadaceae* and the phylum *Proteobacteria* (1). *Comamonas* spp. are commonly found in a variety of environmental samples and are among the common bacteria in soil (2–4), wetlands (5–7), and wastewater (8, 9). The ability of *Comamonas* spp. to catabolize a wide range of organic and inorganic substrates, including amino acids, aromatic compounds, carboxylic acids, heavy metals, and steroids, has been extensively documented (10–12). Furthermore, several studies have documented the roles *Comamonas* spp. can play in wastewater bioaugmentation and bioremediation (13–15). *Comamonas denitrificans* was first identified because of its significant ability to grow in anoxic conditions and denitrify sludge (16, 17), a by-product during sewage treatment rich in antimicrobial residues, metals, pharmaceuticals, and drug-resistant microbial communities (18, 19). Unlike other species in the *Comamonas* genus, *C. denitrificans* can reduce nitrate to nitrogen gas in both aerobic and anaerobic conditions (16) and has a notable capacity of biofilm formation (20).

While *Comamonas* spp. regularly feature in environmental microbial communities, several species have been described as aggressive opportunistic pathogens capable of surviving in hospital environments (21). Reports of *Comamonas* spp. causing disease in humans have been increasing in multiple countries across six continents (22–28). *Comamonas testosteroni* is the species most frequently associated with human disease and is known to cause invasive infections such as cellulitis (29), peritonitis (30), endocarditis (31), meningitis (32), endophthalmitis (33), pneumonia (34), and appendicitis associated with bacteremia (25). However, other *Comamonas* species, such as *Comamonas terrigena* and *Comamonas kerstersii*, can also cause pathologies, including eye and intra-abdominal infections (35, 36). Due to the omnipresence of *Comamonas* spp. in the environment and its apparent capacity to cause opportunistic infection, it is important to understand whether members of the genus display resistance to antibiotics. Antimicrobial resistance (AMR) poses a global public health threat, and the increase in resistance to "last-resort drugs," such as carbapenems, is alarming (37–39). The environment is an important reservoir of resistant bacteria, antimicrobial-resistant genes (ARGs) and antimicrobial residues, and there is mounting evidence suggesting the dispersal of environmental and clinical ARGs between wildlife, agriculture, and humans (40–42). Carbapenem-resistant bacteria have been isolated from natural water bodies (43, 44), wastewater (45–47), wildlife, and synanthropic species that frequent wastewater (42, 48). Airline waste (49) and wastewater in particular has been flagged as a hot spot for AMR evolution given the high abundance of bacteria combined with sublethal antibiotic concentrations derived from anthropogenic sources, including agriculture, industry, hospitals, and households (19, 47).

To date, only one carbapenem resistance gene has been identified in any *Comamonas* spp. (bla_{IMP-8} in *Comamonas thiooxydans*) (50), and in general, data regarding ARG carriage in *Comamonas* are limited. Published case reports suggest that *C. testosteroni* is susceptible to common antibiotics such as aminoglycosides, fluoroquinolones, ceftazidime, carbapenems, and piperacillin-tazobactam (1, 51). However, in a severe meningitis case caused by *C. testosteroni*, the pathogen was resistant to broad spectrum cephalosporin but sensitive to carbapenem (32). *C. denitrificans* has been reported to survive high doses of amoxicillin (52), and a genomic analysis of a *Comamonas aquatica* isolate from the Daguan River, China, identified genes encoding resistance to several antibiotics, including cephalosporins, penicillins, and bacitracin; however, the genes that could account for these resistances were not reported (53).

Given the growing number of studies identifying carbapenem-resistant bacteria in water matrices and the abundance of *Comamonas* in the environment, this study sought to shed light on the potential for *Comamonas* species to acquire genes that encode

TABLE 1 *Comamonas* identification to the species level

Species identification	MALDI-TOF MS	Kraken2[a]	WGS phylogenetic characterization
C. aquatic	1 (2.6%)	12 (30.7%)	1 (2.6%)
C. testosterone	0	6 (15.4%)	5 (12.8%)
C. denitrificans	0	0	33 (84.6%)
C. kerstersii	0	8 (20.5%)	0
Comamonas sp.	25 (64.1%)	2 (5.1%)	0
Nonreliable identification	13 (33.3%)	0	0

[a]Kraken2 typing also resulted in *P. aeruginosa* (n = 1; 2.6%), *Acidovorax carolinensis* (n = 2; 5.1%), and *Acidovorax* spp. (n = 8; 20.5%).

resistance to critically important antibiotics in Australia. Here, we provide a whole-genome sequence (WGS) and phylogenetic analysis of 39 *Comamonas* spp. isolates that grew on Oxoid Brilliance CRE agar plates, including 33 diverse *C. denitrificans* isolates, the majority of which carried acquired chromosomal carbapenemase bla_{GES-5} and β-lactamase bla_{OXA} genes.

RESULTS

From a total of 471 isolates cultured from CRE-agar plates inoculated with samples from aquatic environments in South Australia, including wastewater, wetland, and lake samples from 2018 to 2019, 39 isolates were *Comamonas* spp. *Comamonas* constituted the third most prevalent genus isolated from wastewater and were the subject of this study. Most *Comamonas* spp. were sourced from influent wastewater (n = 37; 94.8%) with single isolates from a lake (n = 1; 2.6%) and a wetland (n = 1; 2.6%). The associated metadata on all 39 isolates described here, as well as 48 *Comamonas* spp. sourced from GenBank, are available in Data Set S1.

Genome assembly. Draft genomes were assembled using Shovill version 1.0.4. Consistent with an earlier whole-genome sequencing study (27), genome sizes ranged between 2,782,493 and 4,055,492 bp with an average size of 3,101,902 bp. The number of scaffolds per genome ranged from 29 to 321, with a mean of 128.9. Full assembly statistics can be viewed in Data Set S2. Draft genomes have been deposited in the NCBI database under the accession numbers SAMN25632339 to SAMN25632377 and BioProject PRJNA803140.

Identification of *Comamonas* species. Identification of *Comamonas* isolates to the species level varied between the typing techniques used (Table 1). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) of the 39 isolates sourced from Australian aquatic environments predicted *Comamonas* sp. for 25 (62.5%) and one *C. aquatica*. Kraken2 predicted 28 isolates as *Comamonas* species (72%), but some were misidentified as *Pseudomonas aeruginosa* (n = 1) and *Acidovorax* spp. (n = 10). Using our phylogenetic approach, all 39 isolates were identified as *Comamonas* spp., specifically 33 *C. denitrificans* (84.6%), 5 *C. testosteroni* (12.5%), and 1 *C. aquatica* (2.5%) isolate.

Phylogenetic analysis. A phylogenetic tree comprising 88 *Comamonas* spp. genomes was constructed using Phylosift (Fig. 1) with 39 isolates from the Australian aquatic environment in this collection and 49 global strains available from GenBank. The isolates were derived from diverse sources, primarily environmental samples (n = 75), humans (n = 5), and various animal waste (n = 5). The count of species postphylogeny were as follows: *C. denitrificans* (n = 34), *C. testosteroni* (n = 19), *Comamonas thiooxydens* (n = 14), *C. aquatica* (n = 4), *C. kerstersii* (n = 2), *Comamonas terrigena* (n = 2), and one isolate each of *Comamonas badia*, *Comamonas terrae*, *Comamonas composti*, *Comamonas granuli*, *Comamonas jiangduensis*, *Comamonas koreensis*, *Comamonas odontotermitis*, *Comamonas piscis*, *Comamonas sediminis*, and *Comamonas serinivorans*. Three isolates could not be characterized to the species level.

Based on the distribution of sequence alignments by PhyloSift, *Comamonas* species were separated into three primary clusters, with a clear separation between species in

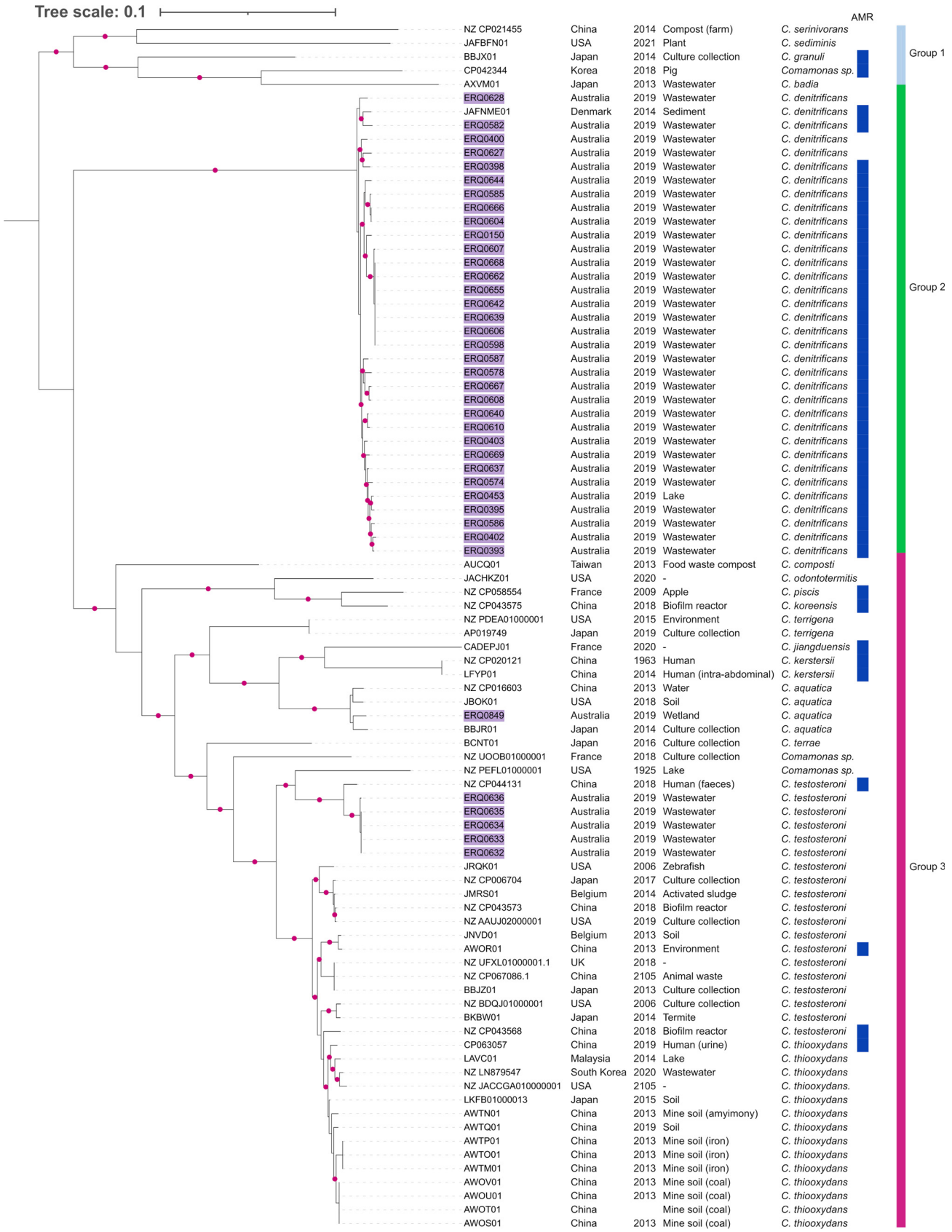

FIG 1 *Comamonas* phylogeny. Midpoint-rooted maximum-likelihood phylogeny and associated metadata of 88 *Comamonas* species using Phylosift. Isolates from this collection are colored in *purple*. Bootstrap values greater than 0.9 are shown as *pink dots*. The *blue strip* designates isolates carrying antimicrobial-resistant genes (ARGs). AMR, antimicrobial resistance.

all cases except *C. testosteroni* and *C. thiooxydans*, which were closely related. *C. denitrificans* was notably separated from other *Comamonas* species, mirroring prior analyses of RNA sequences (54).

Comamonas group 1 (Fig. 1) comprised five species: *C. serinivorans*, *C. sediminis*, *C. granuli*, *C. badia*, and the undefined *Comamonas* sp. strain NLF 7-7 (*Sus scrofa*, South Korea, CP042344). Group 2 was defined as *C. denitrificans* only, including the environmental/wastewater isolates from this study plus the only available *C. denitrificans* genome sequence from GenBank (JAFNME01, from sediment, Denmark). Group 3 constituted isolates across at least 11 species based on current nomenclature, with clade structure suggesting that up to 13 species may be present. Our five *C. testosteroni* isolates were most closely related to *C. testosteroni* strain NFYY023 (NZ_CP044131) from human feces, which sits apart from the *C. testosteroni/C. thiooxydans* subclade; however, *C. testosteroni* strain NFYY023 was ~90,000 single-nucleotide polymorphisms (SNPs) from the closest *C. testosteroni* from this collection. It is likely that our five isolates constitute a closely related yet distinct species to *C. testosteroni*. Using the NCBI *C. testosteroni* representative strain G1 (NZ_CP067086) as a reference, we performed average nucleotide identity BLASTn (ANIb), ANI average nucleotide identity MUMer (ANIm), and *in silico* DNA-DNA hybridization (DDH) analyses to determine whether the isolates belong to *C. testosteroni*. All five isolates had ANIb and ANIm values of 83 and 87%, respectively, which are below the 95% cutoff values for species delimitation, and the *in silico* DDH analysis returned a 0.04% probability of being the same species as NZ_CP067086 (Data Set S3). Definitive novel species characterizations will be the subject of future studies.

To illustrate the more nuanced distribution of *C. denitrificans* in our study, particularly against the representative strain from Denmark, a Parsnp tree was constructed (Fig. 2). First, despite the majority coming from the same Australian wastewater source, we noted a diverse set of clades that generally mirror the clade structure shown in the PhyloSift distribution, including several small subclades of only one to three representatives. Notably, one cluster of isolates (maroon) sat as a very closely related subclade (as low as seven SNPs between eight isolates). The external international reference sat most distant in the distribution, at approximately 6,000 SNPs from both its closest neighbors, suggesting significantly diverged lineages of the species.

Pangenome analysis of *C. denitrificans*. An analysis of the *C. denitrificans* pangenome supported the subclade associations as determined by the core-genome phylogeny and the high genetic diversity of the species (Fig. 3). The *C. denitrificans* pangenome consisted of 7,219 genes, with a core genome of only 946 genes and an accessory genome of 5,173 genes (147 soft-core, 2,637 shell, and 2,389 cloud genes). The distribution of genes generally matched the phylogenetic analysis presented in Fig. 2, with notable divergence in pangenome data for the top-most (light green) and lower two (blue and pink) clades, which had some isolates with distinct genotypes distant from the closest evolutionary neighbors. This comparison aligns with the highly variant nature of the isolates presented here, suggesting that up to 25 distinct lineages of *C. denitrificans* are now available as WGS.

Antimicrobial resistance gene (ARG) identification. All 88 *Comamonas* spp. were screened for ARGs (Fig. 4). In the Australian collection, 30 of the 33 *C. denitrificans* isolates (29 from wastewater, 1 from a lake sample) carried at least one ARG or detergent resistance gene. Among these 33 Australian *C. denitrificans* isolates, 13 carried carbapenemase gene bla_{GES-5}, 9 hosted bla_{OXA-10}, 2 carried $bla_{OXA-101}$, and 2 carried $bla_{OXA-5-like}$ alleles. The bla_{GES} and bla_{OXA} genes were only identified in *C. dentrificans*; therefore, we could not account for resistance to the carbapenem supplement in Oxoid Brilliance CRE Agar plates in 26 of 39 *Comamonas* spp. isolates.

A total of 21 Australian *C. denitrificans* isolates carried at least 1 aminoglycoside resistance gene, with *aadA5* being the most common ($n = 9$) followed by *aadA13* ($n = 6$) (Fig. 4). *C. denitrificans* isolate ERQ0403 carried a combination of clinically important antimicrobial resistance genes (bla_{GES-5} and bla_{OXA-10}) and aminoglycoside resistance (*aadA13*). All but one Australian *C. denitrificans* harbored detergent resistance gene *qacL* (Fig. 4).

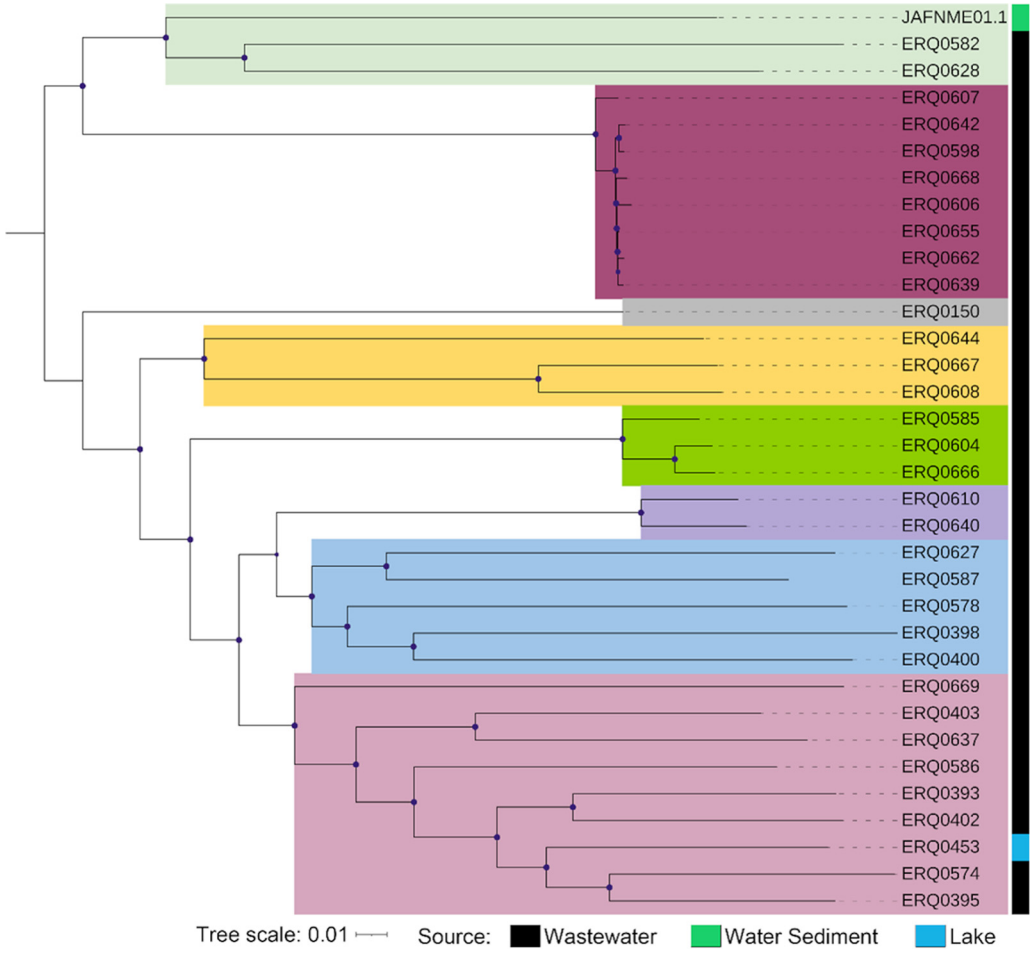

FIG 2 *C. dentrificans* phylogeny. Phylogenetic relationships of Australian *C. denitrificans*. The midpoint-rooted maximum-likelihood tree was generated from a single-nucleotide polymorphism (SNP)-based alignment via Parsnp, with *C. denitrificans* JAFNM01 used as a reference. Clades are colored in descending order: light green, maroon, gray, yellow, green, purple, blue, and pink.

A subset of *Comamonas* isolates sourced from GenBank (n = 11; 22%) also carried ARGs (Fig. 1 and 4), five of which, sourced from either humans or biofilm reactors, had multidrug resistance profiles (Fig. 4). However, outside the Australian *C. denitrificans* isolates, only one isolate, a *C. thiooxydans* sourced from a human sample, carried a carbapenem resistance gene (bla_{IMP-8}).

Metal resistance genes were also identified using the MEGAres 2.0 database. All *C. denitrificans* isolates originating from Australia carried genes conferring resistance to copper (*cop* genes), and 11 (33%) additionally carried mercury resistance genes (*mer* genes). The singular Australian *C. aquatica* isolate also carried copper resistance genes, as did

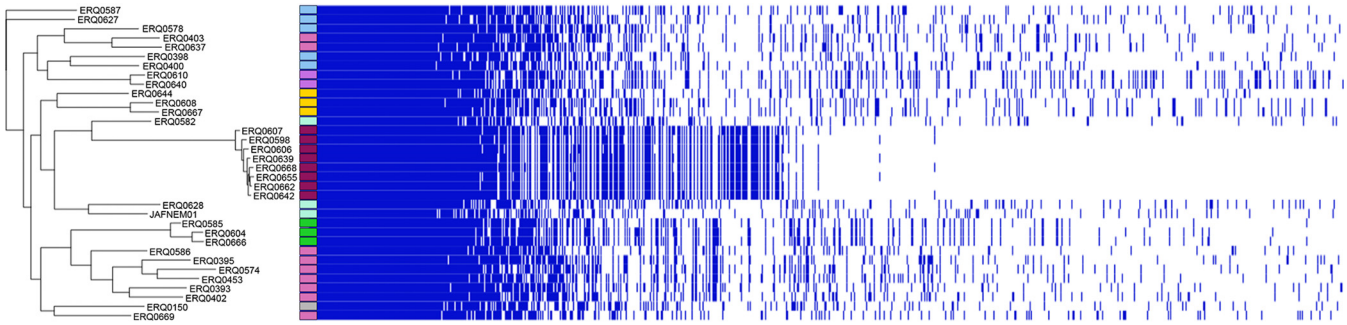

FIG 3 *C. denitrificans* pangenome. Pangenome analyses of 33 *C. denitrificans* from Australian aquatic environments and *C. denitrificans* JAFNEM01 sourced from GenBank. Color groupings match the clades designated in Fig. 2. Phylogenetic clustering is by accessory genomes.

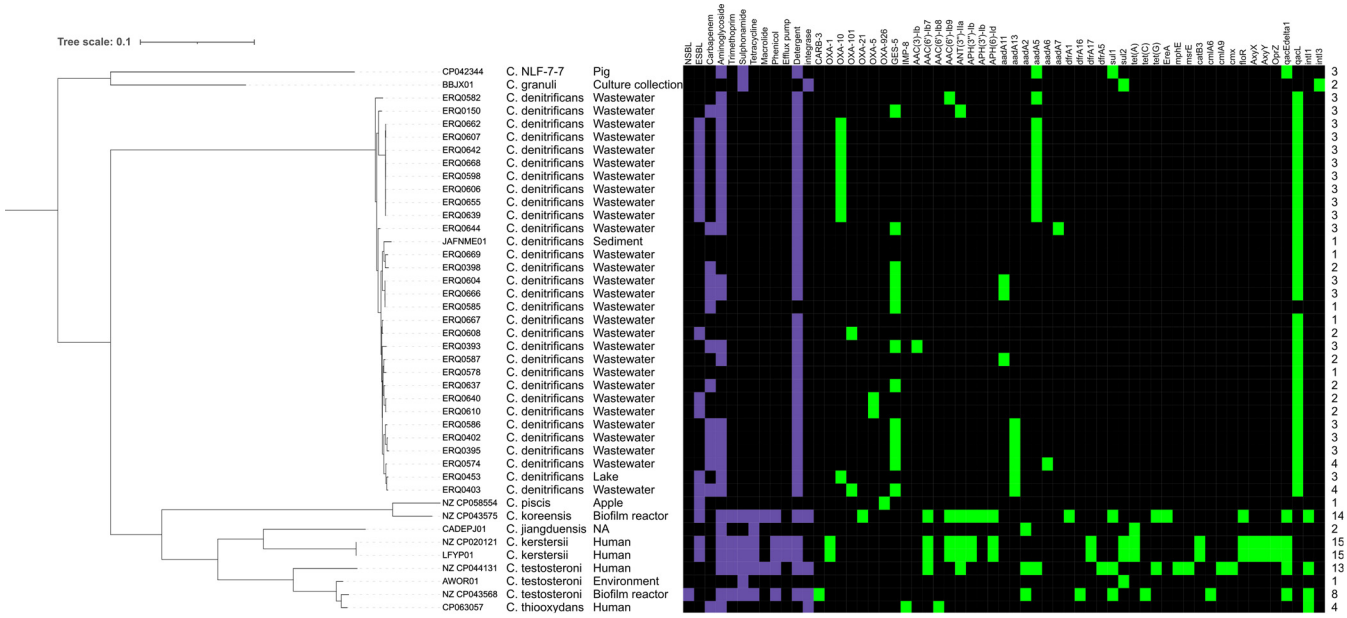

FIG 4 Antimicrobial resistance genes (ARGs) in *Comamonas* spp. Heat map of ARGs identified in all available *Comamonas* spp. genomes adjacent to a midpoint-rooted maximum-likelihood phylogenetic tree generated using Phylosift. Specific ARGs, detergent resistance genes, and integrases are shown in *green*. Resistance conferred by gene carriage is shown in *purple*. NSBL, narrow spectrum β-lactamase.

two *C. aquatica* isolates sourced from GenBank. The five Australian *C. testosteroni* isolates did not carry metal resistance genes; however, eight *C. testosteroni* isolates sourced from GenBank all carried mercury resistance genes, with one isolate carrying an additional gene conferring resistance to chromium. Virulence-associated genes were also screened for using the VFDB; however, none were identified in any isolate using a >80% nucleotide identity and >80% gene coverage cutoff.

(i) *C. denitrificans* chromosomally acquired bla_{GES-5} and bla_{OXA} alleles. Regarding genetic context, bla_{GES-5} and bla_{OXA} genes appeared on putative genomic islands (GIs). Upon comparing the genetic context around bla_{GES-5}, it was determined that two chromosomal sites were hosting the gene, with related isolates ERQ0150, ERQ0395, and ERQ0585 encoding the gene in the same chromosomal loci. One isolate, ERQ0644, had acquired the gene at a separate chromosomal site. Two reference strains (JAFNME01 and ERQ0400) were used to resolve these chromosomal sites across contigs present in the isolates hosting AMR (Fig. 5A and C). In addition to bla_{GES-5}, the GI present in ERQ0395 also harbored *aadA13* (aminoglycoside resistance), *qacL* (quaternary ammonium compound resistance), and several DUF1010 genes. Although the GIs represented in Fig. 5A lacked any tRNA gene or known integrase, they were identified as GIs based on atypical codon usage by SIGI-HMM (55), and most ORFs within the GIs, including all AMR genes, had adjacent *attC* sites, indicating that these ORFs are mobile gene cassettes. The *attC* sites associated with bla_{GES-5} in ERQ0585 and ERQ0150 shared 98% sequence identity but only ~50% sequence identity with the ERQ0395 bla_{GES-5} *attC* site. However, the bla_{GES-5} gene and associated *attC* site in ERQ0395 was identical to that carried by an *intI3*-containing Tn*7221* (also called Tn*Pfu1*; Tn*402* family) in *P. aeruginosa* pIPM3H3-GES5. Unlike the three aforementioned bla_{GES-5}-containing GIs, the GI carrying bla_{GES-5} in ERQ0644, located at a different chromosomal insertion site (Fig. 5C), contained a phage associated integrase *intIPac*, which was also partially present in ERQ0400 (Fig. 5C).

A comparison of two representative GIs from EQR0640 and EQR0655, encoding bla_{OXA-10} and $bla_{OX-5-like}$, respectively, was also visualized (Fig. 5B). Alignments demonstrated that GIs hosting these AMR genes were inserted at the exact same chromosomal site as three GIs containing bla_{GES-5} (Fig. 5A). The shared chromosomal sequence at each structure's termini included *lysR* (transcriptional regulator) and a *fic* family gene, often found in mobile GIs (56) at one end and a partial *lysR* and an *mmsA* (CoA-acylating methylmalonate-semialdehyde

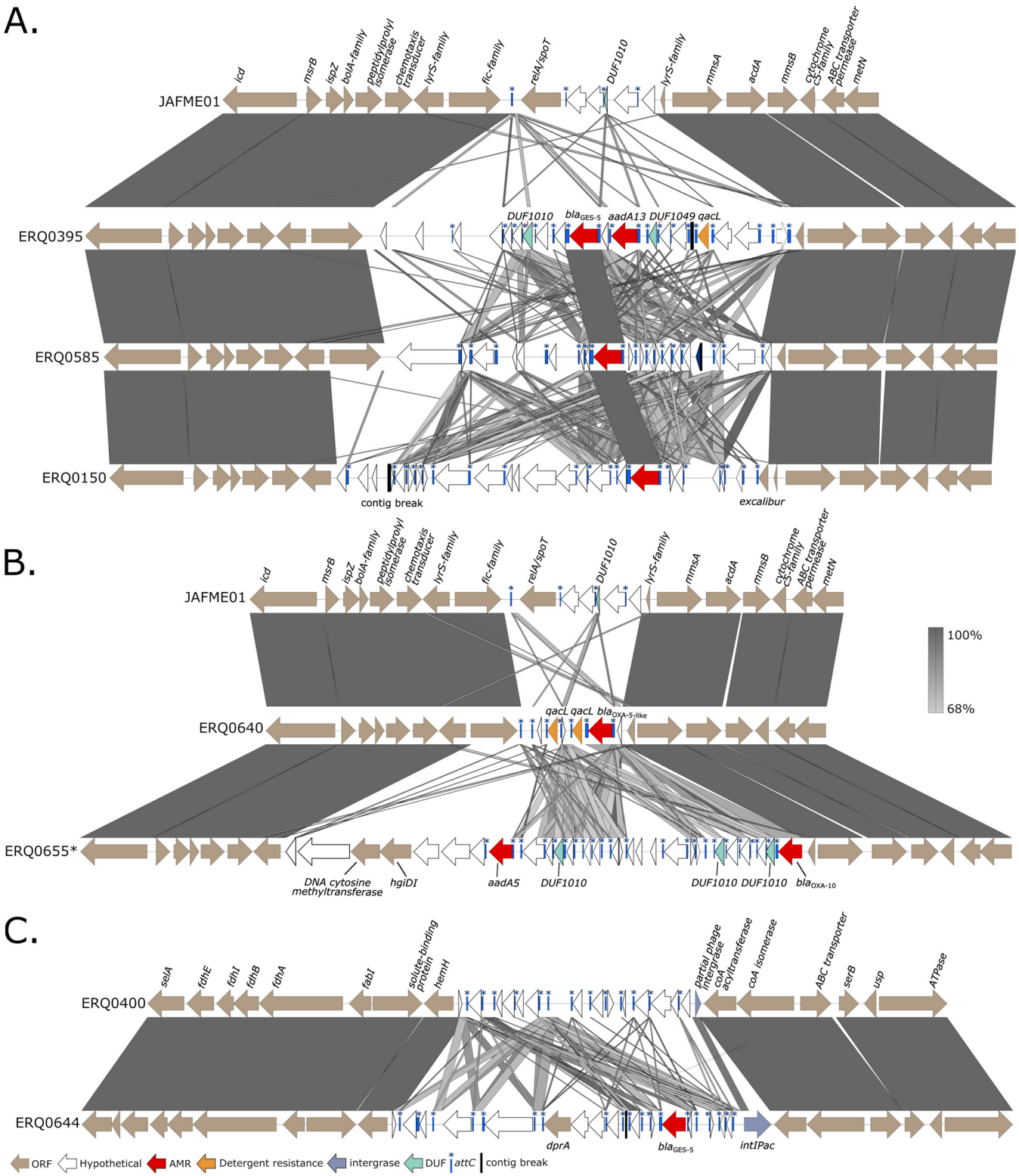

FIG 5 Genomic islands (GIs) carrying AMR determinants. (A) Comparison of three GIs carrying bla_{GES-5} at the chromosomal insertion site. (B) Comparison of two GIs, one carrying bla_{OXA-10}, the other $bla_{OXA-5-like}$. GI in the same genetic context as panel A. (C) Comparison of GI carrying blaGES-5. The genetic context differs to panels A and B and is associated with integrase *intIPac*. ORF, open reading frame.

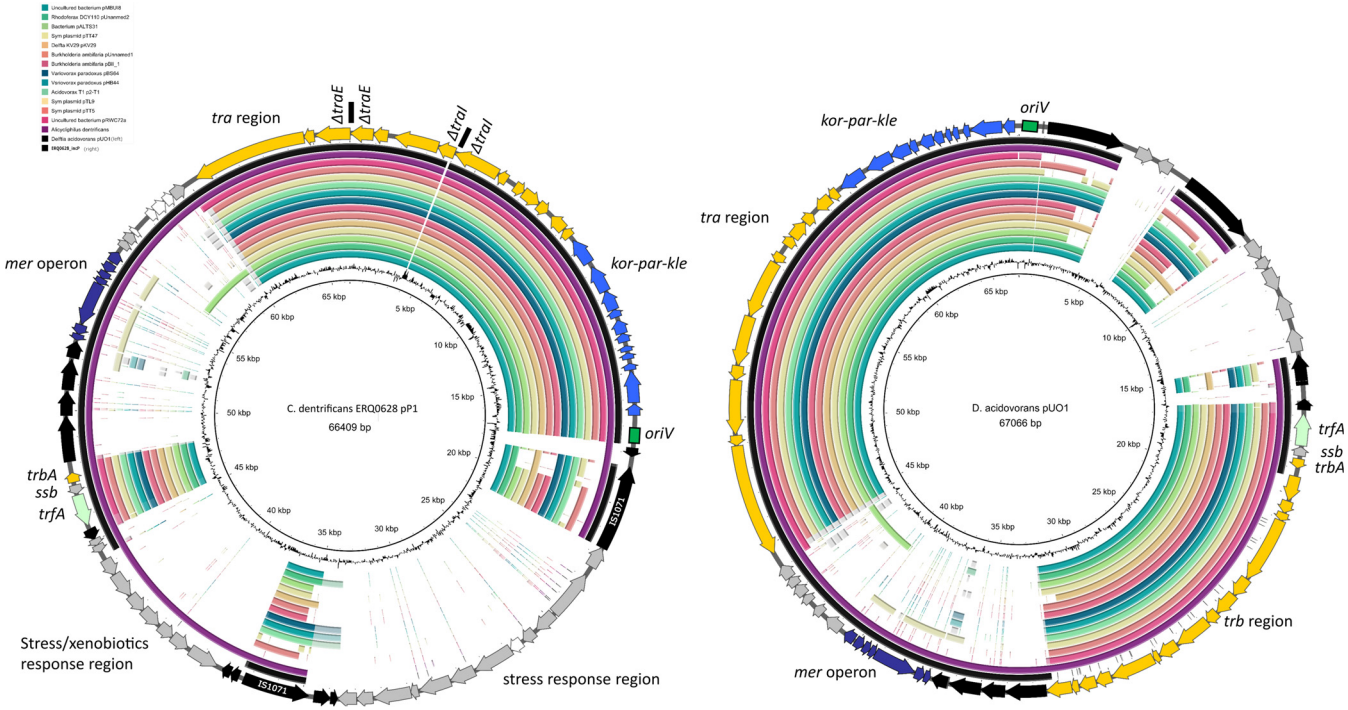

FIG 6 BRIG comparisons of IncP-1 plasmids. (Left) Plasmid map of putative IncP-1 plasmid from ERQ0628 and BLAST alignments to other IncP-1 plasmids. Contig breaks are noted in the *tra* operon. (Right) Plasmid map of *D. acidovorans* pUO1 and BLAST alignments to other IncP-1 plasmids.

dehydrogenase) at the other. Like the bla_{GES-5} GIs, most ORFs, including $bla_{OXA-5-like}$ and two *qacL* genes in EQR0640 and bla_{OXA-10} and *aadA5* in EQR0655, were associated with *attC* sites, although they lacked any known integrase or tRNA gene. All bla_{GES-5}- and bla_{OXA}-containing GIs appear to be unique to this Australian collection of *C. denitrificans*, with no significant homology to GIs previously deposited in GenBank, including a well characterized bla_{GES-5}-containing GI residing in *P. aeruginosa* (57).

***C. denitrificans* IncP-1 plasmid.** All 88 *Comamonas* genomes were screened for plasmid replicons, and three were identified: IncP-1 in *C. denitrificans* isolate ERQ0628 and a *C. jiangduensis* isolate (CADEPJ01), and rep21 in a *C. kerstersii* strain J29 (NZ_LFYP00000000.1). The IncP-1 replicon (*trfA*) in ERQ0628 was located on a 62,429-bp contig, beginning with a partial *traI* gene and ending with a partial *traE* gene. Another contig (3,980 bp) contained both partial *traI* and *traE* genes present at the contig start and end. Future long-read sequencing is needed to investigate any potential additional insertions into the *tra* region following *traI* or *traE*, although previously, insertions have only been reported following *traM*, between *oriV* and *trfA*, and between *trb* and *tra* regions (58). Nevertheless, a BLAST search of the NCBI database was used to identify high sequence identity IncP-1 plasmids, and the top 15 hits (ranging between 98.78 and 99.95% sequence identity and 40 and 79% sequence coverage) were used for content comparisons (Fig. 6).

All compared IncP-1 plasmids possessed an IncP-type conjugation *tra* region and the IncP *kor-par-kle* regulon region. The remaining regions present in ERQ628 IncP-1 (Fig. 6, left) were less common. An 11,709-bp inserted region containing a mercury resistance *mer* operon carried by Tn*5031* (Tn*402* family) was identified in two other similar plasmids, one carried by *Alicycliphilus denitrificans* pALIDE02 (CP002451) and the other by *Delftia acidovorans* pUO1 (AB063332) (59). These two Tn*5031* sequences were nearly identical, differing by 1 SNP. *A. denitrificans* pALIDE02 was the only other plasmid to possess a 7,964-bp inserted region containing genes involved in stress response and xenobiotic degradation, including two genes encoding glutathione *S*-transferases and a short-chain dehydrogenase/reductase (SDR). The ERQ628 IncP-1 plasmid also possesses a unique 12,843-bp inserted region flanked by two IS*1071* elements and contained genes involved in oxidative stress response,

including *msrA*, *msrB*, and *ydiU*. In *D. acidovorans* pUO1 (Fig. 6, right), IS*1071* elements flank genes responsible for haloacetate degradation (*dehH1* and *dehH2*). Notably, the ERQ0628 IncP-1 plasmid only contained *trbA*, and the remaining *trb* region was missing (Fig. 6, right).

DISCUSSION

Wastewater has been flagged as a hot spot for interaction between diverse bacteria, genetic exchange, and AMR evolution and dissemination across ecosystems (60). However, despite the abundance of *Comamonas* species in wastewater, its ability to acquire AMR is poorly understood. Here, we added 39 draft *Comamonas* genomes, sourced from South Australian municipal wastewater, a lake, and a wetland, to make up a total pool of 88 *Comamonas* WGS currently available. Our analyses show that (i) carbapenem-resistant *Comamonas* was most prevalent in wastewater, (ii) *C. denitrificans* isolates carried genomic islands encoding carbapenemase bla_{GES-5} or extended-spectrum β-lactamase (ESBL) bla_{OXA} alleles, (iii) *Comamonas* carry genes conferring resistance to copper and mercury, (iv) *C. denitrificans*, the most commonly isolated species, was genetically diverse, and (v) a *C. denitrificans* isolate carried an IncP-1 plasmid with genes involved in xenobiotic degradation and response to oxidative stress.

Carbapenem-resistant *Comamonas* is present in aquatic environments: implications for public health and wastewater treatments. Carbapenem-resistant Gram-negative bacteria are an urgent threat to global health (61, 62) and have been detected in both wastewater and surface water worldwide (46, 63–65). Here, we isolated 39 carbapenem-resistant *Comamonas* isolates: 32 *C. denitrificans* and 5 *C. testosteroni* from wastewater, 1 *C. denitrificans* from a wetland, and 1 *C. aquatica* from a lake with public access. To the best of our knowledge, this is the first report of carbapenem resistance in *C. denitrificans* and *C. aquatica*, although there has been at least one report of a carbapenem-resistant *C. testosteroni* infection in Turkey in 2015 (66). Infections caused by carbapenem-resistant bacteria are associated with poor prognoses and increased morbidity and mortality rates (67). *C. testosteroni* has been isolated as the infectious agent in various sites, including blood, peritoneal fluid, cerebrospinal fluid, urine, and different tissues (68). *C. aquatica* reportedly has caused bacteremia and septic shock (69), and *C. denitrificans* has been detected in clinical samples (26). Therefore, the emergence of carbapenem resistance in these organisms warrants closer public health surveillance. To that end, it is important to note that *Comamonas* spp. grow poorly on routine clinical media (26), and biochemical tests often provide erroneous *Pseudomonas* spp. identifications (1). Similarly, as shown here and elsewhere, MALDI-TOF MS can misidentify *Comamonas* at the species level (70). Furthermore, *C. denitrificans* and *Comamonas nitrativorans* have highly similar 16S rRNA sequences and cannot be differentiated by phenotypic analysis (1). Conversely, WGS represents a high-resolution method that can accurately identify *Comamonas* species.

The presence of carbapenem-resistant *Comamonas* in wastewater also has implications for wastewater treatments. Wastewater treatment plants receive large volumes of sewerage enriched in inorganic constitutes, such as metals and chemicals, and organic constituents, such as feces and food waste (71, 72). Biological treatment of wastewater using microbes is effective and easy to implement at a low cost, particularly when using indigenous microbial communities (73). A recent meta-analysis of 20 diverse metagenomic wastewater samples across 11 countries found that *Comamonas* spp. was dominant in industrial, biological, and municipal wastewater (74). In wastewater, *Comamonas* spp. play important roles in the degradation of benzene, ethylbenzene, and toluene (75); dye (13); naphthalene-2-sulfonic acid (76); organics (14); pyridine (15); and quinoline and phenol (77) and in the denitrification (78) and bioremediation of petrochemicals (79). These capabilities, in addition to high biofilm-forming properties, make *Comamonas* good candidates for biofilm systems wastewater treatment (80, 81). However, a recent study demonstrated that spiking wastewater biofilm reactors with streptomycin and oxytetracycline caused the acquisition of class 1 integron integrases (*intI1*) and *aadA* genes (aminoglycoside resistance) in *Comamonas* strains (82). Given this recent observation, it is perhaps unsurprising that genes conferring resistance to aminoglycosides were identified in 30 *C. denitrificans*

isolates (91%) in our collection, with *aadA5* the most prevalent ($n = 9$), and while *intI1* was not identified in this collection, the potential for capture should not be overlooked. On that note, bacteria in wastewater exist as heterogeneous communities, and the presence of *Comamonas* is positively correlated with the presence of *Acinetobacter*, *Aeromonas*, and *Pseudomonas*, suggesting that these microbes form a symbiotic relationship (74). Horizontal gene transfer occurs in proximal bacteria, and all three species are known producers of carbapenemases (46, 83). Therefore, a symbiotic relationship with these organisms may not only involve the cooperative degradation of pollutants but also the exchange of AMR determinants.

***C. denitrificans* carry genomic islands encoding either bla_{GES-5} or bla_{OXA} alleles.** Genes encoding carbapenemases confer resistance to carbapenems, penicillins, cephalosporins, and monobactams, thus severely limiting treatment options (67). While all 39 *Comamonas* isolates described here had a carbapenem-resistant phenotype, only 13 isolates, all *C. denitrificans*, carried a known carbapenemase gene, bla_{GES-5}, suggesting novel AMR mechanisms for the remaining isolates. The bla_{GES-5} gene cassette has previously been detected in the genera of *Citrobacter*, *Enterobacter*, *Escherichia*, *Leclercia*, and *Lelliottia* found in wastewater (46, 63, 84); in *Citrobacter* and *Klebsiella* in surface water (44, 85) and was commonly associated with class 1 and 3 integrons. The gene has also been detected in clinical *P. aeruginosa* strains carried by plasmids and genomic islands (57, 86, 87).

Genomic islands are fragments of DNA inserted into the chromosome via horizontal gene transfer and play an important role in the evolution and adaptation of bacteria through the dissemination of ARGs and virulence genes and the formation of catabolic pathways (88). We were able to resolve four examples in which a bla_{GES-5} gene cassette was located on predicted genomic islands, and in one instance, the bla_{GES-5} gene cassette was identical to that carried by *intI3*-containing Tn*7221* structure found on a *P. aeruginosa* plasmid. However, genomic islands are generally characterized by atypical G+C content and codon usage and the presence of integrons and are typically inserted at tRNA loci (89). Of the four putative genomic islands, only one carried an integrase, *IntIPac*, previously reported promoting AMR in *Acidithiobacillus ferrooxidans* (90). Apart from atypical G+C content and codon usage, the remaining three genomic islands did not contain any typical genomic island characteristics and appeared to consist of a collection of gene cassettes located in the same chromosomal loci. Curiously, we also resolved two predicted genomic islands, one carrying ESBL bla_{OXA-10}, commonly found in *Acinetobacter baumannii* (91), and the other a novel $bla_{OXA-5-like}$ gene, both located at the same chromosomal loci as the three bla_{GES-5} containing genomic islands. Although rare, gene cassettes have been found outside an integron context (92); however, the mechanisms behind their insertion have not been determined.

***Comamonas* spp. carry heavy metal and biocide resistance genes.** In addition to residual antibiotics, it has been postulated that the high AMR levels in water matrices are also due to the presence of metals and biocides, which can exert selective pressure for ARGs through coselection (93). Additionally, previous studies have observed a correlation between metal resistance and β-lactamase production in bacteria, particularly in regard to mercury resistance (94). Although we did not find any apparent relationship between ARG, heavy metal, and biocide carriage, we did identify 11 *C. denitrificans* isolates (33%) that carried mercury resistance genes (*mer* genes), and genes conferring resistance to copper (*cop* genes) were present in all *C. denitrificans* and the singular *C. aquatica* isolate in this collection. Additionally, 29 *C. denitrificans* isolates (89%) carried detergent resistance gene *qacL*. Interestingly, a study on the association of metal tolerance with antibiotic susceptibility in *C. acidovorans* found that mercury-resistant phenotypes were resistant to nitrofuran, β-lactams, aminoglycosides, glycopeptides, and tetracycline, while copper-resistant phenotypes were susceptible to all tested antibiotics (95). However, ARG carriage was not determined.

IncP-1 plasmid present in *C. denitrificans*. Sensing stressful conditions and adjusting cellular metabolism accordingly are essential for bacteria to survive in variable environments such as wastewater (96). IncP-1 plasmids are promiscuous self-

transmissible plasmids with broad host ranges and an ability to swiftly acquire and transfer genes involved in the degradation of introduced xenobiotics (97). IncP-1 plasmids have been previously identified in *Comamonas* spp. isolated from wastewater that were involved in the degradation of dyes (98). However, only one IncP-1 replicon was identified in *Comamonas* from Australian aquatic environments: a *C. denitrificans* isolate from wastewater. The IncP-1 replicon was situated on a putative 66,409-bp plasmid containing three inserted regions. One inserted region contained a functional *mer* operon, a well characterized metal resistance system capable of degrading highly toxic mercury into volatile, nontoxic forms (9). Another inserted region carried genes involved in oxidative stress response and was flanked by IS*1071* elements. IS*1071* is known to flank many catabolic genes in diverse Gram-negative and Gram-positive bacteria (99) and has been shown to transpose at high frequencies in *C. testosteroni* (100). The last inserted region carried two glutathione *S*-transferases, known to degrade a wide range of toxic chemicals, including carcinogens, environmental pollutants, and oxidative stress products (101), and a short-chain dehydrogenase/reductase, involved in the metabolism of aromatic hydrocarbons, including steroids and sugars (99). Unlike most, if not all, IncP-1 plasmids, the majority of *trb* genes, which form the sex pili, were missing in the *C. denitrificans* IncP-1 plasmid, meaning that in all probability, it is nonconjugative.

Concluding remark. At the time of this study, only one *C. denitrificans* genome was publicly available (JAFNME01). Thus, this study has contributed significantly to our understanding of the genetic diversity of this species. Our phylogenetic and pangenome analyses identified at least 25 distinct *C. denitrificans* lineages, and *C. denitrificans* JAFNME01 isolated from sediment in Denmark in 2019 was approximately 6000 SNPs from its closest neighbor from this collection. Acquisition of the critically important antibiotic resistance genes bla_{GES-5} and bla_{OXA} on genomic islands and an IncP-1 plasmid carrying metal resistance is a serious cause for concern and should be monitored given the important role *C. denitrificans* plays in bioremediation and wastewater treatment. This may require: (i) improvements in growth medium to ensure reliable culture; (ii) more reliable diagnostic assays, including improvements to MALDI-TOFF species identification databases; and (iii) introduction of long-read metagenomic sequencing strategies to monitor wastewater. Finally, we could not identify the genetic basis for carbapenem resistance in a significant subset of the *Comamonas* isolates described here. Further studies are required to rectify this knowledge gap.

MATERIALS AND METHODS

Sample collection and bacterial isolation. Within a period of 1 year, from July 2018 to July 2019, water samples (~10 L) were collected in triplicate, monthly, from three sources: influent wastewater, a lake, and a wetland in South Australia.

Influent wastewater was collected from four municipal wastewater treatment plants (here referred to as WWTPs A, B, C, and D). WWTP A serves ≈150,000 inhabitants and treats approximately 33 ML/day of primarily domestic sewage. WWTP B serves ≈198,000 inhabitants and treats around 55 ML/day, primarily from households and commercial establishments with minor industrial inputs. WWTP C serves ≈700,000 inhabitants and treats around 175 ML/day with a large industrial/commercial component, as well as residential and hospital sources. WWTP D is a rural wastewater treatment plant that serves 5,000 inhabitants and treats around 1.2 ML/day primarily from households and seasonal meat-processing facilities. Raw wastewater to all WWTPs is classified as low-to-medium organic strength (102). All samples were stored on ice directly after collection and processed within 2 to 3 h.

The lake is a shallow artificial lake fed by recycled water, which is composed of a mix of treated wastewater and cleansed storm water. The lake is sporadically fringed by aquatic reads and vegetation and attracts silver gulls (*Larus novaehollandiae*), pigeons (*Columba livia*), Eurasian coots (*Fulica atra*), and indigenous and migratory birds.

The inland wetland covers 42 ha and is recharged by seasonal rainfall/runoff inflows. The annual mean rainfall is 438 mm (11.4 mm in February; 59.6 mm in July) and mean annual temperature ranging between 21.6°C (28.2°C in January) and 11.5°C (7.1°C in July) (bom.gov.au). The wetland is habitat to over 160 species of indigenous and migratory birds, including purple swamp hem, stilt, and herons, as well as *L. novaehollandiae*, *F. atra*, *C. livia*, and *Haliaeetus leucogaster*. The water of this inland wetland is harvested and pumped underground into natural sandy limestone aquifers.

Isolation of carbapenem-resistant *Comamonas* species and MALDI-TOF MS species identification. All of the samples were plated, in triplicate, on Oxoid Brilliance CRE agar plates (Thermo Fisher Scientific Australia, Adelaide, SA, Australia) after 10-fold serial dilutions, using 500 μL from 2 to 3 consecutive dilutions. Oxoid Brilliance CRE agar plates are chromogenic screening plates selective for carbapenem-resistant

Enterobacteriaceae (CRE). The formulation contained a modified carbapenem at a level recommended by both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI). The two-chromogen system differentiated *Escherichia coli* (pale pink) from the KESC group organisms (*Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter*) (blue). Other carbapenem-resistant organisms not from the *Enterobacteriaceae* family (e.g., *Acinetobacter*) produced white or naturally pigmented colonies. All cultures were incubated at 25, 37, and 44°C for 24 h. Single colonies growing on CRE agar were picked up and steaked on a plate counting agar (PCA; Thermo Fisher Scientific). Then, PCA cultures were incubated at 37°C for 18 to 24 h to have sufficient bacterial growth. A total of 40 *Comamonas* isolates were sampled, with their identity resolved initially by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS was performed using Bruker Daltonics, operated in linear positive mode under MALDI Biotyper 3.0 real-time classification (version 3.1, Bruker Daltonics) (103). All isolates were preserved in glycerol (40% v/v) at −80°C. Clinical *Klebsiella pneumoniae* and *C. testosteroni* carbapenem resistance strains were used as controls to ensure the isolation conditions and that carbapenem plates were efficient and reproducible. The control strains were identified by MALDI-TOF, bla_{KPC}, bla_{GES}, and bla_{OXA} qPCR assays (104) and WGS.

DNA extraction and WGS. DNA from MALDI-TOF MS identified isolates (scores ≥ 2-3) were extracted using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. Nucleic acid quality was measured with Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA concentrations for all samples were measured by fluorometric quantitation using a Qubit instrument and high sensitivity double-stranded DNA HS assay kit (Thermo Fisher Scientific), and purified DNA extracts were stored at −80°C until sequencing.

WGS was performed on an Illumina Nextseq500 instrument according to the manufacturer's instructions. The data were uploaded to Basespace (www.basespace.illumina.com), which converted the raw data to eight FASTQ files for each sample. Genome assembly was performed using Shovill version 1.0.4 (https://github.com/tseemann/shovill) using the SPAdes option. A single genome was cut due to high scaffolding, with the remaining 39 sequences passing basic quality control using assembly stats (github.com/sanger-pathogens/assembly-stats).

Phylogenetic analysis. A phylogenetic analysis was performed on *Comamonas* isolates from multiple species, including: 39 draft genomes from aquatic environments in South Australia (this study) alongside 49 *Comamonas* genomes (22 complete and 27 draft) sourced from GenBank (downloaded on 5 March 2021) (105) (ncbi.nlm.nih.gov/GenBank/) including five clinical *Comamonas* isolates. GenBank sourced genomes are referred to by their accession numbers throughout this article. A maximum-likelihood phylogenetic tree of all species were constructed using PhyloSift (106), and SNP-based phylogenetic analyses were generated using Parsnp (107) (github.com/marbl/parsnp). All trees were resolved using FastTree2 (108) and visualized using Interactive Tree Of Life (iTOL) software version 4 (109) (itol.embl.de/). The *C. denitrificans* pangenome was calculated using Roary version 3.11.2 (110) (github.com/sanger-pathogens/Roary) and visualized using Phandango version 1.3.0 (111) (jameshadfield.github.io/phandango/#/main). A pangenome-wide gene association study on *C. denitrificans* isolates was performed using Scoary (112) (github.com/AdmiralenOla/Scoary).

Genotyping. In addition to MALDI-TOF MS, *in silico* species identification was also performed using Kraken2 (113) (ccb.jhu.edu/software/kraken2/). To confirm the identification of *C. denitrificans* and *C. testosteroni*, pairwise genome comparisons were performed against *C. denitrificans* JAFNME01 and *C. testosteroni* NZ_CP067086.1 using both the ANIb and ANIm algorithms available on the JSpecies web server (jspecies.ribohost.com/jspeciesws) using a 95% cutoff value for species delimitation (114). Predicted DDH results were ascertained using the Genome-to-Genome Distance Calculator (GGDC) tool (115) (ggdc.dsmz.de) with a 70% cutoff value for species delimitation using the recommended Formula 2. Virulence-associated genes, antimicrobial resistance (AMR) genes, metal resistance genes, and plasmid replicons were screened for using ABRicate (github.com/tseemann/abricate) in conjunction with the following databases: VFDB (116) (mgc.ac.cn/VFs), CARD (117) (card.mcmaster.ca), MEGARes 2.0 (118) (megares.meglab.org), and PlasmidFinder (119) (cge.cbs.dtu.dk/services/PlasmidFinder), respectively.

Genome annotation. All draft environmental genomes in this study were annotated using prokka (github.com/tseemann/prokka) and managed using SnapGene version 4.1.9 (snapgene.com). The RAST annotation pipeline (120) (rast.nmpdr.org/rast.cgi) was also utilized on genomes representative of each clade to cross-check annotations. Transposons were identified using TnCentral (tncentral.proteininformationresource.org). Putative genomic islands (GIs) were identified by Islandviewer 4 (121) (pathogenomics.sfu.ca/islandviewer/) using reference genome *Comamonas* sp. strain NLF 7-7 (accession NZ_CP042344.1) and confirmed in detail using progressive Mauve (122). Mobile gene cassette-associated recombination (*attC*) sites were screened for using HattCI (github.com/maribuon/HattCI) (123). Only *attC* sites with V scores greater than 7.5 were considered. GIs were visualized using EasyFig (124) (mjsull.github.io/Easyfig/). BLASTn was utilized to determine whether putative GIs and AMR regions identified in this study had been previously deposited into NCBI. The Aliview software version 3.0 (GPLv3) (125) (github.com/AliView), utilizing MUSCLE and FastTree2 was used to align gene alleles and perform maximum-likelihood phylogenetic analyses. Plasmid comparisons were performed using the BLAST Ring Image Generator (BRIG) (126) (http://brig.sourceforge.net/).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

ACKNOWLEDGMENTS

We respectfully acknowledge the Peramangk, Kaurna, and Ngarrindjeri people as the traditional owners and custodians of the waters and lands where the environmental samples used in this research were collected.

Also acknowledge Gianluca Brunetti for helping with the water sample collection and processing and Robin Lockington for bacterial culturing and isolation.

We declare no conflict of interest.

S.H.: writing – original draft, formal analysis, investigation, writing – review & editing; E.R.W.: investigation, methodology, validation, conceptualization, supervision, writing – review & editing; B.D.: investigation, conceptualization, validation, formal analysis, data curation, writing – review & editing; E.D.: conceptualization, funding acquisition, supervision, writing – review & editing; I.G.C.: investigation, resources; D.J.B.: resources, methodology; V.M.J.: writing – original draft, formal analysis, investigation, supervision, conceptualization, writing – review & editing; S.P.D.: conceptualization, resources, supervision, funding acquisition, project administration, writing – review & editing.

This research was partly supported by Australian Government Medical Research Future Fund Project MRFF75873 and the Australian Centre for Genomic Epidemiological Microbiology (Ausgem), a collaborative research initiative between the New South Wales Department of Primary Industries and the University of Technology Sydney; and by Quadram Institute Bioscience Biotechnology and Biological Sciences Research Council (BBSRC)-funded Core Capability Grant Project BB/CCG1860/1 and BBSRC Institute Strategic Program Microbes in the Food Chain Project BB/R012504/1.

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