1 Title Page

2 Emergence of a novel mobile RND-type efflux pump gene cluster,
3 *tmexC3D2-toprJ1b*, in *Pseudomonas* species

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23 #Equal contribution

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25 Running title: A novel *tmexC3D2-toprJ1b* gene cluster in *Pseudomonas*

26

27 Abstract

28 Tigecycline exhibits promising activity against multidrug-resistant
 29 Gram-negative bacteria (MDR-GNB), whose infections are difficult to treat with
 30 antimicrobials. However, mobile tigecycline resistance genes, such as
 31 *tmexCD-toprJ*, have emerged in Enterobacterales isolated in China, Vietnam,
 32 and possibly other countries in the world. In this study, we investigated
 33 tigecycline-nonsusceptible GNB in Japan. Eight tigecycline- and
 34 carbapenem-nonsusceptible isolates of *Pseudomonas alcaligenes* were
 35 obtained from sewage water from a medical institution in Japan in 2020. Whole
 36 genome analysis of all *P. alcaligenes* isolates was performed using short-read
 37 sequencing, and the isolate KAM426 was further analyzed using long-read
 38 sequencing. For important antimicrobial resistance genes, analysis of
 39 surrounding structures and comparison with similar sequences in the public
 40 genome database were performed. We identified a novel hybrid type of
 41 *tmexCD-toprJ* gene cluster, *tmexC3D2-toprJ1b* consisting of *tmexC3*, *tmexC2*,
 42 and *toprJ1b*, in phylogenetically clonal isolates of *P. alcaligenes*. The complete
 43 genome sequence of KAM426 revealed that this isolate co-harbors
 44 *tmexC3D2-toprJ1b* and two copies of the carbapenemase gene *bla_{IMP-1}* on the
 45 chromosome. *tmexC3D2-toprJ1b* in KAM426 was flanked by the IS5/IS1182
 46 family transposase gene, suggesting that the gene cluster was acquired by
 47 horizontal gene transfer (HGT). *tmexC3D2-toprJ1b* seemed to have spread to
 48 other *Pseudomonas* species such as *Pseudomonas aeruginosa* via HGT
 49 mediated by mobile gene elements such as a plasmid. This study identified
 50 *tmexCD-toprJ*-like tigecycline resistance genes in Japan for the first time and

51 suggests that diverse *tmexCD-toprJ*-like gene clusters, including
52 *tmexC3D2-toprJ1b*, have spread among MDR-GNB worldwide. Further
53 epidemiological genomic studies in clinical and environmental settings are
54 needed.

55

56 Introduction

57 Tigecycline is considered a last-resort antimicrobial against infections caused
58 by MDR Gram-negative bacteria. Recently, mobile tigecycline resistance genes,
59 *tet(X3)*, *tet(X4)*, and other variants, *tet(X5)* to *tet(X15)*, encoding
60 flavin-dependent monooxygenases that catalyze tigecycline degradation have
61 emerged in Enterobacterales and *Acinetobacter* species in China and other
62 countries.¹⁻⁴ Furthermore, mobile tigecycline resistance gene clusters,
63 *tmexCD1-toprJ1*, *tmexCD2-toprJ2*, and *tmexCD3-toprJ1b* (also designated as
64 *tmexCD3-toprJ3*), encoding the resistance–nodulation–cell division (RND) efflux
65 pumps that excrete multiple antimicrobials, including tetracyclines such as
66 tigecycline, cephalosporins, fluoroquinolones, and aminoglycosides, have
67 emerged predominantly in Enterobacterales in China and Vietnam.⁵⁻¹¹ Mobile
68 *tmexCD-toprJ* genes are estimated to originate from chromosomal *mexCD-oprJ*
69 genes in *Pseudomonas* species.⁵ Interestingly, however, *tmexCD1-toprJ1* has
70 also been shown to spread to *Pseudomonas putida* by a megaplasmid.⁷

71 Here, we report *Pseudomonas alcaligenes* isolates harboring a novel variant
72 of *tmexCD-toprJ* along with two copies of a metallo-β-lactamase (MBL) gene,
73 *bla_{IMP-1}*. *P. alcaligenes* is a Gram-negative aerobic rod belonging to the bacterial
74 family Pseudomonadaceae, of which members are common inhabitants of soil
75 and water and are rare opportunistic human pathogens.¹² *P. alcaligenes* has
76 also been suggested to be a causative agent of secondary bacterial infection
77 during COVID-19 pneumonia.¹³ However, little is known about the clinical
78 importance of *P. alcaligenes*, mainly because of the difficulties in identifying and
79 distinguishing this bacterium from closely related *Pseudomonas* species such as

80 *P. aeruginosa*, *P. mendocina*, and *P. pseudoalcaligenes*, in clinical settings.

81

82 Results and discussion

83 Eight cephalosporin-resistant isolates of *P. alcaligenes* were obtained from
84 medical wastewater in Japan in 2020. Whole-genome sequence analysis of *P.*
85 *alcaligenes* isolates by Illumina HiSeq X and the core genome phylogeny based
86 on their draft genome sequences showed that these isolates were
87 phylogenetically very similar (Fig. S1). Moreover, all *P. alcaligenes* isolates
88 harbored the same set of antimicrobial resistance (AMR) genes, including
89 *tmexCD-toprJ*-like genes, *bla*_{IMP-1} (MBL gene conferring carbapenem resistance),
90 *aac(6')-Ib-cr* (aminoglycoside resistance gene), *fosE* (fosfomycin resistance
91 gene), *qacG2* (multidrug resistance gene), and *sul1* (sulfonamide resistance
92 gene), suggesting that these isolates were clonally disseminated (Fig. S1).

93 One of the *P. alcaligenes* isolates, KAM426, was further sequenced using
94 ONT MinION, and hybrid sequence analysis using both Illumina and ONT reads
95 resulted in the circular complete chromosome sequence (4.68 Mb, accession no.
96 [AP024354](#)). Average nucleotide identity (ANI) analysis revealed that KAM426 is
97 96.5% identical to *P. alcaligenes* strain NCTC 10367^T (type strain, accession no.
98 [UGUP00000000](#)), and the isolate harbored *tmexCD-toprJ*-like genes along with
99 two copies of *bla*_{IMP-1} on its chromosome (Fig. 1). Also, *P. alcaligenes* KAM426
100 harbored a set of T4SS- and T6SS-associated genes, respectively, that would
101 be potential virulence factors (Fig. 1). AST showed that *P. alcaligenes* KAM426
102 was nonsusceptible to tigecycline and broad-spectrum β -lactams, including
103 carbapenems. According to the broth dilution method, the minimal inhibitory
104 concentration (MIC) of tigecycline against KAM426 was 2 mg/L and was
105 decreased to 1 mg/L in the presence of the efflux pump inhibitor NMP. According

106 to the Etest, the MICs of imipenem and meropenem against KAM426 were 8 and
107 >32 mg/L, respectively, and these were decreased to <1 and 0.19 mg/L,
108 respectively, in the presence of the MBL inhibitor EDTA. These results
109 suggested that *tmexCD-toprJ*-like genes and *bla_{IMP-1}* would be responsible for
110 tigecycline and carbapenem resistance in this isolate. In *P. aeruginosa*,
111 chromosomal efflux pump genes such as *mexCD-oprJ* (putative ancestor genes
112 of *tmexCD-toprJ*) are known to contribute to tigecycline resistance.⁵ Though *P.*
113 *alcaligenes* KAM426 did not harbor *mexCD-oprJ*-like genes on the chromosome,
114 other chromosomal efflux pump genes could contribute tigecycline resistance in
115 addition to *tmexCD-toprJ*-like genes.

116 The coding sequence of the *tmexCD-toprJ*-like gene cluster in *P. alcaligenes*
117 KAM426 (1,999,524 to 2,005,275 nt in accession no. [AP024354](#)) was highly
118 identical to that of *tmexCD1-toprJ1* in *Klebsiella pneumoniae* strain AH581
119 [96.8% (5,569/5,753 nt), 70,998 to 76,749 nt in accession no. [MK347425](#)]
120 isolated from livestock in China in 2017,⁵ that of *tmexCD2-toprJ2* in *Raoultella*
121 *ornithinolytica* strain NC189 [98.4% (5,660/5,752 nt), 182,964 to 188,715 nt in
122 accession no. [MN175502](#)] isolated from a human in China in 2018,⁸ and that of
123 *tmexCD3-toprJ1b* in *Proteus terrae* subsp. *cibarius* strain SDQ8C180-2T [98.1%
124 (5,644/5,753 nt), 3,321,781 to 3,327,532 nt in accession no. [CP073356](#)] isolated
125 from a chicken in China in 2018,¹¹ respectively. The identities of the *tmexC*-like
126 gene in KAM426 (KAM426_19240) compared with *tmexC1*, *tmexC2*, and
127 *tmexC3* were 94.0% (1,094/1,164 nt), 94.6% (1,101/1,164 nt), and 98.5%
128 (1,147/1,164 nt, the gene product was 98.4% identical to TMexC3 with six amino
129 acid substitutions), respectively. For the *tmexD*-like gene in KAM426

130 (KAM426_19250), these identities were 96.5% (3,025/3,136 nt), 99.1%
 131 (3,108/3,135 nt, the gene product was perfect match to TMexD2), and 97.1%
 132 (3,046/3,136 nt), respectively. For the *toprJ*-like gene in KAM426
 133 (KAM426_19260), the identities were 99.9% (1,433/1,434 nt), 99.9%
 134 (1,417/1,419 nt), and 100% (1,434/1,434 nt, the gene product was perfect match
 135 to TOprJ1b), respectively. Thus, we designated *tmexCD-toprJ*-like genes in
 136 KAM426 as *tmexC3D2-toprJ1b*.

137 The *tnfxB*-like gene, which has been suggested to be involved in the
 138 expression of the *tmexCD-toprJ*-like gene,⁵ was found upstream
 139 *tmexC3D2-toprJ1b* in *P. alcaligenes* KAM426 (KAM426_19230 in accession no.
 140 [AP024354](#)). The identities of the *tnfxB*-like gene in KAM426 compared with
 141 *tnfxB1*, *tnfxB2*, and *tnfxB3* (accession nos. [MK347425](#), [CP054471](#), and
 142 [CP073356](#)) were 97.7% (545/558 nt), 97.8% (540/552 nt), and 99.8% (557/558
 143 nt, the gene product was 99.5% identical to TNfxB3 with one amino acid
 144 substitution), respectively. Thus, we designated the *tnfxB*-like gene in KAM426
 145 as *tnfxB3*. *tnfxB3-tmexC3D2-toprJ1b* was flanked by the IS5/IS1182 family
 146 transposase gene (Fig. 2A upper). Furthermore, the genomic region containing
 147 *tnfxB3-tmexC3D2-toprJ1b* in KAM426 was surrounded by many putative mobile
 148 gene elements (MGEs), and this genomic region was not present in *P.*
 149 *alcaligenes* strain NEB 585 (accession no. [CP014784](#)) (Fig. 2A). NEB 585 was
 150 isolated from a water environment in the United States in 1989 and is the only
 151 other *P. alcaligenes* strain for which the complete chromosome sequence has
 152 been reported¹⁴ other than KAM426. The genomic recombination regions in
 153 KAM426 and NEB 585 encoded a set of common genes (KAM426_19430 to

154 KAM426_19470 in accession no. [AP024354](#), and AOT30_13575 to
155 AOT30_13555 in accession no. [CP014784](#)) (Fig. 2A), although their functions
156 are unknown. The results suggest that KAM426 acquired *tmexCD-toprJ*-like
157 genes, which confer resistance to multiple antimicrobials including tigecycline,
158 via horizontal gene transfer (HGT) mediated by MGEs.

159 BLASTn analysis using megablast revealed that two *Pseudomonas* species
160 strains in the NCBI database of Nucleotide collection (nr/nt) have the exact same
161 sequence containing the *tmexC3D2-toprJ1b* gene cluster along with *nfxB*. A
162 *bla*_{DIM-2}-harboring *Pseudomonas* species strain, BJP69, isolated from a human
163 in China in 2015¹⁵ carries *tmexC3D2-toprJ1b* on its chromosome (accession no.
164 [CP041933](#)) and a *bla*_{KPC-2}-harboring *P. aeruginosa* strain, NDTH9845, isolated
165 from a human in China in 2018 carries *tmexC3D2-toprJ1b* on its IncP-2
166 megaplasmid, pNDTH9845 (accession no. [CP073081](#)) (Fig. 2B). ANI analysis
167 confirmed that BJP69 is 98.0% identical to *Pseudomonas juntendi* strain BML3^T
168 (type strain, accession no. [BLJG01000000](#)) and that NDTH9845 is 99.2%
169 identical to *P. aeruginosa* DSM 50071^T (type strain, accession no.
170 [FUXR01000000](#)). *tmexC3D2-toprJ1b* was determined to be flanked by the
171 IS5/IS1182 family transposase gene in *P. aeruginosa* pNDTH9845, whereas no
172 MGE was found upstream or downstream of *tmexC3D2-toprJ1b* in *P. juntendi*
173 BJP69 (Fig. 2B). Together with *P. alcaligenes* KAM426 in this study, these three
174 *Pseudomonas* species strains harbor acquired carbapenemase genes, in
175 addition to *tmexC3D2-toprJ1b*, showing that they have accumulated clinically
176 relevant AMR genes. Of note, *P. juntendi* BJP69 carried one more copy of
177 *tmexCD-toprJ*-like genes, *tmexC2D2-toprJ2*, on its IncP-2 megaplasmid,

178 pBJP69-DIM (accession no. [MN208064](#)).

179 The integron-integrase *IntI1* catalyzes site-specific recombination between the
180 *attI1* and *attC* sites.¹⁶ The class 1 integron gene cassette consisting of *intI1* with
181 the *attI1* site, *qacEΔ1* (disrupted form of *qacE*), and *sul1* in *P. alcaligenes*
182 KAM426 (accession no. [AP024354](#)) contain several AMR genes, including *fosE*,
183 two copies of *aac(6')-Ib-cr*, *bla_{IMP-1}*, and *qacG2* with their *attC* sites (Fig. 3A). The
184 *bla_{IMP-1}*-containing integron gene cassette in KAM426 was found to be
185 surrounded by many putative transposase genes, and this MGE-containing
186 genomic region was not present in *P. alcaligenes* NEB 585 (accession no.
187 [CP014784](#)) (Fig. 3A). The genomic region around ATP-dependent helicase
188 genes (KAM426_37950 and KAM426_36180 in accession no. [AP024354](#), and
189 A0T30_05350 in accession no. [CP014784](#)) could be a hot spot for HGT (Fig. 3A),
190 but there have been no reports to suggest this possibility to date.

191 The other copy of *bla_{IMP-1}* was contained within a partial structure of the
192 integron gene cassette consisting of two copies of *aac(6')-Ib-cr* followed by
193 *bla_{IMP-1}*, as described previously herein (Fig. 3A), in a different location in the
194 chromosome of *P. alcaligenes* KAM426 (Fig. 3B). Interestingly, a comparison
195 between the *bla_{IMP-1}*-containing genomic region of *P. alcaligenes* KAM426 and
196 the corresponding genomic region in *P. alcaligenes* NEB 585 revealed the
197 presence of multiple copies of short repeated sequences (approximately 80 bp),
198 which were identical to PARs (*Pseudomonas alcaligenes* repetitive DNAs) within
199 the super-integron In55044 in *P. alcaligenes* strain ATCC 55044 (accession no.
200 [AY038186](#))¹⁷ (Fig. 3B).

201 The super-integron, which was first identified in *Vibrio cholerae*, is

distinguished from conventional integrons in several respects, such as size and the nature of the genes contained within cassettes and contributes to the acquisition of AMR genes.¹⁸⁻²⁰ The PARs were reported as recombination sites for the integrase gene (*intI*_{Pac} in accession no. [AY038186](#)) in the super-integron in *P. alcaligenes*.¹⁷ The PARs in *P. alcaligenes* KAM426 and *P. alcaligenes* NEB 585 contained conserved sequences of inverted repeats (1L, 2L, 2R, and 1R) with a PAR signature and variable regions between inverted repeats 2L and 2R, as shown in *P. alcaligenes* ATCC 55044¹⁷ (Fig. S2). Although KAM426 and NEB 585 lacked the integrase gene flanking the PAR and most of the contained genes had no known function, these plastic genomic regions in both strains retained their evolutionary histories of gene acquisitions mediated by the super-integron. There was no PAR around *bla*_{IMP-1} in KAM426, suggesting that the super-integron is not directly involved in the acquisition of *bla*_{IMP-1}, and this genomic region is likely one of the hot spots for HGT. KAM426 is thought to have incorporated *bla*_{IMP-1} into the class 1 integron gene cassette first (Fig. 3A) and then incorporated the partial structure containing *bla*_{IMP-1} into the other genomic region flanked by the super-integron (Fig. 3B), leading to a high level of resistance by increasing the copy number of AMR genes.

Our study provides a glimpse into environmental bacteria that have been rapidly and silently becoming resistant to clinically relevant antimicrobials, including tigecycline and carbapenem, and highlights the importance of AMR monitoring using wastewater to detect a future clinical crisis before it happens. Furthermore, *P. alcaligenes* would be considered an important environmental reservoir that supplies AMR genes to other related *Pseudomonas* species that

226 are more virulent and likely to cause nosocomial infections, such as *P.*
227 *aeruginosa*.

228 **Materials and methods**

229 **Bacterial isolation and antimicrobial susceptibility testing**

230 Eight cephalosporin-resistant isolates of *P. alcaligenes* (KAM426, KAM428,
231 KAM429, KAM430, KAM432, KAM434, KAM435, and KAM436) were obtained
232 from sewage water from a medical institution in Japan in February, 2020.
233 Environmental water samples were collected and cultured using DHL
234 (Deoxycholate Hydrogen sulfide Lactose) agar containing 2 mg/L of ceftriaxone.
235 Bacterial species identification was performed using MALDI Biotyper (Bruker).
236 antimicrobial susceptibility testing (AST) using *Escherichia coli* ATCC 25922 as
237 quality control was performed according to the broth dilution method based on
238 the CLSI 2020 guidelines or according to the Etest (bioMérieux) based on the
239 manufacturer instructions. For tigecycline, AST was additionally performed in the
240 presence or absence of 75 mg/L of the efflux pump inhibitor
241 1-(1-naphthylmethyl)-piperazine (NMP) as used in the previous study.⁵

242 243 **Whole-genome sequencing and subsequent bioinformatics analysis**

244 Whole-genome sequencing of all eight cephalosporin-resistant isolates of *P.*
245 *alcaligenes* was performed using HiSeq X (Illumina), and the isolate KAM426
246 was further sequenced using MinION [Oxford Nanopore Technologies (ONT)]
247 with the R9.4.1 flow cell. The library for Illumina sequencing (paired-end, insert
248 size of 500-900 bp) was prepared using Nextera XT DNA Library Prep Kit and
249 the library for ONT sequencing was prepared using Rapid Barcoding Kit
250 (SQK-RBK004). Illumina reads were assembled de novo using Shovill v1.1.0
251 (<https://github.com/tseemann/shovill>) with default parameters, resulting in the

252 draft genome sequences. For KAM426, ONT reads were basecalled using
253 Guppy v4.2.2 with the high-accuracy mode, and then both Illumina and ONT
254 reads were assembled de novo using Unicycler v0.4.8.0
255 (<https://github.com/rwick/Unicycler>) with default parameters, resulting in the
256 complete circular chromosome sequence (accession no.: [AP024354](https://www.ncbi.nlm.nih.gov/nuclseq/CP024354)).

257 Coding sequence (CDS) annotation and average nucleotide identity (ANI)
258 analysis were performed using the DFAST server (<https://dfast.nig.ac.jp>).
259 Antimicrobial resistance (AMR) genes were detected using ResFinder v4.1
260 (<http://www.genomicepidemiology.org>) with default parameters using the
261 customized AMR gene database including all known *tmexCD-toprJ* genes. Type
262 IV secretion system (T4SS)- and type VI secretion system (T6SS)-associated
263 genes were detected using TXSScan v1.0.5
264 ([https://research.pasteur.fr/en/tool/txsscan-models-and-profiles-for-protein-secre](https://research.pasteur.fr/en/tool/txsscan-models-and-profiles-for-protein-secretion-systems/)
265 [tion-systems/](https://research.pasteur.fr/en/tool/txsscan-models-and-profiles-for-protein-secretion-systems/)). Circular genomic sequence was visualized using the CGView
266 server (<http://cgview.ca>). Linear comparison of sequence alignment was
267 performed using BLAST and visualized using Easyfig v.2.2.2
268 (<http://mjsull.github.io/Easyfig/>).

269

270 **Nucleotide Sequences**

271 The complete genome sequence of *P. alcaligenes* KAM426 has been
 272 deposited at GenBank/EMBL/DDBJ under the accession number [AP024354](#).
 273 Draft genome sequences of *P. alcaligenes* KAM428, KAM429, KAM430,
 274 KAM432, KAM434, KAM435, and KAM436 have been deposited at
 275 GenBank/EMBL/DDBJ under the accession numbers [BPMN000000000](#),
 276 [BPMP000000000](#), [BPMQ000000000](#), [BPMR000000000](#),
 277 [BPMS000000000](#), and [BPMT000000000](#), respectively.

278

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- 348

349 **Legends**

350 Fig. 1. Circular representation of the chromosome of *Pseudomonas alcaligenes*
 351 KAM426 (accession no. [AP024354](#)) harboring *tmexC3D2-toprJ1b*, along with
 352 two copies of *bla*_{IMP-1} (shown in Figs. 2 and 3), isolated in Japan in 2020. Gray,
 353 green, purple, black, red, yellow, cyan, light green, and orange indicate coding
 354 sequences (CDS), GC skew+, GC skew-, GC content, tigecycline or
 355 carbapenem resistance genes (TRG/CRG), other AMR genes (ARG), mobile
 356 gene elements (MGE), type IV secretion system (T4SS)-associated genes, and
 357 type VI secretion system (T6SS)-associated genes, respectively.

358
 359 Fig. 2. The *tmexC3D2-toprJ1b* gene cluster in *Pseudomonas alcaligenes*
 360 KAM426. (A) Genetic context of the *tmexC3D2-toprJ1b* gene cluster in *P.*
 361 *alcaligenes* KAM426 and its surrounding genomic region (the region between
 362 1,977,208 and 2,027,209 nt in accession no. [AP024354](#)), and structural
 363 comparison with the corresponding genomic region in *P. alcaligenes* NEB 585
 364 (the region between 2,990,265 and 2,915,762 nt in accession no. [CP014784](#)).
 365 (B) Structural comparison of the *tmexC3D2-toprJ1b* gene cluster in *P.*
 366 *alcaligenes* KAM426 (the region between 1,995,475 and 2,006,928 nt in
 367 accession no. [AP024354](#)) with that in the chromosome of *P. juntendi* BJP69 (the
 368 region between 3,340,041 and 3,349,533 nt in accession no. [CP041933](#)) and in
 369 plasmid pNDTH9845 of *P. aeruginosa* NDTH9845 (the region between 225,014
 370 and 215,640 nt in accession no. [CP073081](#)). The strain names of *Pseudomonas*
 371 species, along with the country and year in which bacteria were isolated, are
 372 shown. *tmexC3D2-toprJ1b* genes (TRG), other AMR genes (ARG), mobile gene

elements (MGE), and other genes (Other) are highlighted in red, yellow, light blue, and gray, respectively. Sequence identity is shown as a color scale with the indicated percentages.

376

Fig. 3. Two copies of *bla*_{IMP-1} genes in *Pseudomonas alcaligenes* KAM426. (A) Genetic context of the class I integron gene cassette containing *bla*_{IMP-1} in *P. alcaligenes* KAM426 and its surrounding genomic region (the region between 3,757,827 and 4,033,855 nt in accession no. [AP024354](#)), and structural comparison with the corresponding genomic region in *P. alcaligenes* NEB 585 (the region between 1,159,724 and 1,200,667 nt in accession no. [CP014784](#)). (B) Genetic context of the partial integron gene cassette containing the other *bla*_{IMP-1} gene in *P. alcaligenes* KAM426 and its surrounding genomic region (the region between 2,181,279 and 2,188,592 nt in accession no. [AP024354](#)), and structural comparison with the corresponding genomic regions in *P. alcaligenes* NEB 585 (the region between 2,758,939 and 2,768,635 nt in accession no. [CP014784](#)) and in *P. alcaligenes* ATCC 55044 (super-integron In55044 in accession no. [AY038186](#)). The strain names of *P. alcaligenes*, along with the country and year in which bacteria were isolated, are shown. *bla*_{IMP-1} genes (CRG), other AMR genes (ARG), mobile gene elements (MGE), other genes (Others), and *P. alcaligenes* repetitive DNA (PAR) are highlighted in red, yellow, light blue, gray, and khaki green, respectively. Sequence identity is shown as a color scale with the indicated percentages.

395

Fig. S1. Core genome phylogeny constructed by Roary v3.13.0

397 (<https://github.com/sanger-pathogens/Roary>) with minimum percentage identity
398 for BLASTp=70% and RAxML v8.2.4
399 (<https://github.com/stamatak/standard-RAxML>), with 1,000 bootstraps using
400 ceftriaxone-resistant *Pseudomonas alcaligenes* isolates in this study and
401 reference strains of *P. alcaligenes* and *P. aeruginosa* (NCTC 10367^T and NEB
402 585 for *P. alcaligenes*, and PAO1 for *P. aeruginosa*). *P. aeruginosa* was used as
403 the outgroup. Bar lengths represent the number of substitutions per site in the
404 core genome. Detected AMR genes, genome assembly status (complete or draft
405 genome sequence, contig numbers if draft), sizes, and accession numbers are
406 shown.

407

408 Fig. S2. Alignment of *Pseudomonas alcaligenes* repetitive DNAs (PARs) in *P.*
409 *alcaligenes* strains. One PAR in *P. alcaligenes* ATCC 55044 (super-integron
410 In55044 in accession no. [AY038186](#)), two PARs in *P. alcaligenes* KAM426
411 (accession no. [AP024354](#)), and 11 PARs in *P. alcaligenes* NEB 585 (accession
412 no. [CP014784](#)) are shown. The multiple alignment comparison was performed
413 and visualized using MAFFT v7 (<https://mafft.cbrc.jp/alignment/software/>). The
414 PAR signature sequence and variable region are shown. Open boxes and
415 arrows represent consensus sequences of inverted repeats (1L, 2L, 2R, and 1R),
416 as described previously.¹⁷

Fig. 1

Fig. 2

A

P. alcaligenes

KAM426

(1,977,208 - 2,027,209 nt)
Japan, 2020

P. alcaligenes

NEB 585

(2,990,265 - 2,915,762 nt)
USA, 1989

B

P. alcaligenes

KAM426

(1,995,475 - 2,006,928 nt)
Japan, 2020

P. juntendi

BJP69

(3,340,041 - 3,349,533 nt)
China, 2015

P. aeruginosa

pNDTH9845

(225,014 - 215,640 nt)
China, 2018

■ TRG ■ ARG ■ MGE ■ Other

Fig. 3

A

P. alcaligenes

KAM426

(3,757,827 - 4,033,855 nt)
Japan, 2020

P. alcaligenes

NEB 585

(1,159,724 - 1,200,667 nt)
USA, 1989

B

P. alcaligenes

KAM426

(2,181,279 - 2,188,592 nt)
Japan, 2020

P. alcaligenes

NEB 585

(2,758,939 - 2,768,635 nt)
USA, 1989

P. alcaligenes

ATCC 55044

(super-integron In55044)
USA

■ CRG ■ ARG ■ MGE ■ Other ● PAR