

AKADÉMIAI KIADÓ

European Journal of  
Microbiology and  
Immunology



14 (2024) 2, 210-218

DOI:  
10.1556/1886.2023.00066  
© 2024 The Author(s)

ORIGINAL RESEARCH  
PAPER



# Clonal dissemination of *Acinetobacter radioresistens* among Humboldt penguins (*Spheniscus humboldti*) inhabiting a barren northern Peruvian island

Ignasi Roca<sup>1,2</sup>, Kathya Espinoza<sup>3,4</sup>, Cinthia Irigoien-Lovera<sup>5</sup>, Maria Piquet<sup>1</sup>, Luciano A. Palomino-Kobayashi<sup>3,4</sup>, Angie K. Castillo<sup>3,4</sup>, Diego D. Gonzales-DelCarpio<sup>5</sup>, Joaquim Viñes<sup>1,6</sup>, Laura Muñoz<sup>1,2</sup>, Barbara Ymaña<sup>3,4</sup>, Rosario Oporto<sup>3,4</sup>, Carlos Zavalaga<sup>5</sup>, Maria J. Pons<sup>3,4\*</sup>  and Joaquim Ruiz<sup>3,4\*\*</sup> 

<sup>1</sup> Department of Microbiology, Biomedical Diagnostic Center (CDB) and ISGlobal, Hospital Clínic – Universitat de Barcelona, Barcelona, Spain

<sup>2</sup> CIBER de Enfermedades Infecciosas (CIBERINFEC), Instituto de Salud Carlos III, Madrid, Spain

<sup>3</sup> Grupo de Investigación en Dinámicas y Epidemiología de la Resistencia a Antimicrobianos – “One Health”, Universidad Científica del Sur, Lima, Peru

<sup>4</sup> Grupo de Investigación en Enfermedades Emergentes y Reemergentes, Universidad Científica del Sur, Lima, Peru

<sup>5</sup> Unidad de Investigación de Ecosistemas Marinos-Grupo Aves Marinas, Universidad Científica del Sur, Lima, Peru

<sup>6</sup> Servei Veterinari de Genètica Molecular (SVGGM), Universitat Autònoma de Barcelona, Barcelona, Spain

Received: December 24, 2023 • Accepted: February 27, 2024

Published online: March 14, 2024

## ABSTRACT

*Acinetobacter* spp. are often isolated from natural sources, but knowledge about their presence in wild animals is fragmented and incomplete. The present study aimed to characterize a series of *Acinetobacter radioresistens* isolated from Humboldt penguins (*Spheniscus humboldti*). Fifteen Humboldt penguins from an inhabited northern Peruvian island were sampled. Microorganisms were identified by MALDI-TOF MS. Antibiotic susceptibility to 12 antimicrobial agents was established, and clonal relationships were determined. A representative isolate was selected for whole genome sequencing (WGS). *A. radioresistens* were isolated from the feces of 12 (80%) Humboldt penguins, being susceptible to all the antimicrobial agents tested, except eight cefotaxime-intermediate isolates. All *A. radioresistens* were clonally related. WGS showed that the isolate belonged to ST1972, the presence of two chromosomal encoded carbapenemases (*bla*<sub>OXA-23</sub> and a putative subclass B3 metallo- $\beta$ -lactamase), and a series of point mutations in antibiotic-resistance related chromosomal genes, which were considered as polymorphisms. In addition, a few virulence factors, including a capsule-encoding operon, superoxide dismutases, catalases, phospholipases and a siderophore receptor were identified. The present results suggest that *A. radioresistens* may be a common member of the gut microbiota of Humboldt penguins, but further studies in other geographical areas are needed to establish this finding.

## KEYWORDS

*Acinetobacter* spp., one health, South America, antibiotic susceptibility, OXA-23

\*Corresponding author. Antigua Panamericana Sur Km 19, Villa El Salvador, 15067-Lima, Peru.  
Tel.: +(51) 1 610 6400.  
E-mail: [mpons@cientifica.edu.pe](mailto:mpons@cientifica.edu.pe), [ma.pons.cas@gmail.com](mailto:ma.pons.cas@gmail.com)

\*\*Corresponding author. Antigua Panamericana Sur Km 19, Villa El Salvador, 15067-Lima, Peru.  
Tel.: +(51) 1 610 6400.  
E-mail: [jruizb@cientifica.edu.pe](mailto:jruizb@cientifica.edu.pe), [jorui.trabajo@gmail.com](mailto:jorui.trabajo@gmail.com)



## INTRODUCTION

Several members of the genus *Acinetobacter* are often isolated as a cause of human diseases, with *Acinetobacter baumannii* being the most commonly identified in hospital settings [1, 2]. Nevertheless, most of the recognized *Acinetobacter* species are commonly recovered from other sources, including marketed food, pets, surfaces or wild animals [3–7].

*Acinetobacter radioresistens* was first identified in cotton and soil samples in 1988 [6]. *A. radioresistens* is a rare cause of human infections, with a role as opportunistic pathogen having been confirmed [8, 9]. Of note, *A. radioresistens* from human infections often present high antibiotic susceptibility levels [8–10], different to that reported for *A. baumannii* [1, 2, 11, 12].

It is remarkable that the OXA-23 group, a common cause of carbapenem resistance in *A. baumannii* [1, 13], is considered intrinsic of this microorganism [14]. Nevertheless, in *A. radioresistens*, this gene usually does not result in carbapenem resistance, and it has been proposed that in the absence of an upstream insertion sequence, such as IS*Acra1*, that may provide a strong promoter sequence, it is not expressed, or is expressed at low basal levels [14, 15].

Outside clinical settings, *A. radioresistens* has been isolated from other sources, such as inanimate surfaces, human skin or Antarctic soils [13, 16, 17]. Regarding animals, *A. radioresistens* has been recovered from different human-related animal species, such as cats or dogs, horses and chickens [4, 7, 18], as well as exotic pets, including chameleons, tortoises or geckos, among others [19], being scarcely reported in wild animals, such as Fitzinger's robber frog (*Craugastor fitzingeri*) or yellow-legged gulls (*Larus michahellis*) [18, 20].

In this scenario, the present study aimed to characterize a series of *A. radioresistens* recovered from Humboldt Penguins (*Spheniscus humboldti*) on an uninhabited guano inshore island in northern Peru.

## MATERIAL AND METHODS

### Sampling area

The sampling was conducted in November–December 2019 on Guañape Norte Island (8°32'41"S, 78°57'49"W). Guañape Norte is a barren rocky island spanning 0.34 km<sup>2</sup>, situated 16 km off the Peruvian coasts within the *Reserva Nacional Sistema de Islas, Islotes y Puntas Guaneras* (a Peruvian Marine Protected Area). The island is uninhabited, except for two permanent rangers stationed there. However, it is visited by hundreds of guano harvesters at intervals of 4–5 years, with the last guano campaign dating back to 2014. Meanwhile, access to the island for researchers is restricted to occasional brief *ad-hoc* visits. Since 2019, small-scaled touristic boats have sporadically approached the island, but landing is prohibited (CZ, personal communication).

The island serves as a significant seabird breeding site, with an estimated population of 1802 Humboldt Penguins,

coexisting with thousands of Guanays (*Leucocarbo bougainvilliorum*) and Peruvian Boobies (*Sula variegata*). Additionally, the presence of sea mammals, including the South American Sea Lion (*Otaria byronia*), is commonly observed on the island [21].

### Humboldt penguins

Fifteen Humboldt Penguin chicks undergoing rearing were carefully captured for the study by gently extracting them from their nests using a noose attached to a 2-m pole. The noose was delicately secured around their necks and pulled out, allowing the penguins to be restrained by hand [22, 23]. The entire handling process took less than 1 min. Cloacal swabs were collected, transported to the laboratory (12–14 days) and cultured as previously described [3].

### Culture and identification

Recovered microorganisms were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a MALDI Biotyper Sirius instrument (MALDI Biotyper<sup>®</sup>, Bruker Daltonics GmbH & Co. KG, Bremen, Germany) running in positive linear mode and using the MBT Compass v4.1 software package and the MBT Compass reference library (version 2022) for taxonomic identification. When more than one microorganism belonging to the same species was recovered from the same sample, only one was considered for further analysis.

### Detection of *bla*<sub>OXA-23</sub>

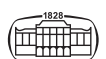
The presence of the *bla*<sub>OXA-23</sub> group gene (thereafter noted as *bla*<sub>OXA-23G</sub>) was established by polymerase chain reaction following standard procedures [1]. The *bla*<sub>OXA-23G</sub>-producing the *A. baumannii* 308 isolate was used as a positive control [24].

### Antimicrobial resistance

Antimicrobial resistance levels to cefotaxime, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, levofloxacin, tetracycline and tigecycline were established by disk diffusion in accordance with the Clinical & Laboratory Standards Institute (CLSI) guidelines [25]. Meanwhile, colistin resistance was established by agar-spot as previously described [26]. In the absence of CLSI/European Committee on Antimicrobial Susceptibility Testing/Food and Drug Administration criteria [25, 27, 28], the tigecycline susceptibility breakpoints considered were; susceptible  $\geq 16$  mm, intermediate 13–15 mm, resistant  $\leq 12$  mm as proposed by Jones et al. [29].

### Clonal relationships

Clonal relationships were established by pulsed field gel electrophoresis (PFGE) and by Fourier-transform infrared spectroscopy (FTIR). PFGE was performed as described previously [30], using genomic digestions with the *ApaI* restriction enzyme and a CHEF-DRIII system (Bio-Rad



Laboratories, Hercules, CA, USA). Band patterns were compared with the InfoQuest™ FPv.5.4 software (Bio-Rad Laboratories, Hercules, CA, USA) and the unweighted pair group method with arithmetic mean was used for cluster analysis based on the Dice similarity coefficient. Using bandwidth tolerance and optimization values set at 1.5 and 1%, respectively, isolates were considered to belong to the same PFGE cluster (pulsetype) if their Dice similarity index was  $\geq 85\%$  [31].

For FTIR spectroscopy, infrared absorption spectra were captured using a Bruker IR Biotyper instrument (Bruker Daltonic, GmbH & Co. KG, Bremen, Germany) running on the IR Biotyper software version 3.1 and on default settings (32 scans per technical replicate; spectral resolution,  $6\text{ cm}^{-1}$ ; apodization function, Blackman-Harris 3-term; zero-filling factor, 4). Measurements were carefully checked and spectra that did not meet the quality criteria ( $0.4 < \text{absorption} < 2$ ; signal-to-noise ratio,  $>40$ ; fringes [ $\times 10^{-6}$ ],  $<100$ ) were removed from the analysis. Spectra were acquired within the  $4,000\text{--}400\text{ cm}^{-1}$  wavenumber range but spectra processing for initial clustering of the isolates were based on the second derivative of the  $1,300\text{--}800\text{ cm}^{-1}$  region that is mostly associated with absorption data from cell wall carbohydrates. Cluster analysis was performed using Euclidean distances and the UPGMA linkage algorithm.

Both PFGE and FTIR cluster analysis included three clonally-related OXA-23-producing *A. baumannii* isolates for outgroup comparison [12].

## DNA extraction and sequencing

DNA was extracted using the ZymoBIOMICS™ DNA miniprep Kit (Zymo Research, Irvine, USA), according to the manufacturer's protocol. DNA quality and quantity were determined using the NanoDrop™ 2000 Spectrophotometer and the Quantus™ Fluorometer and QuantiFluor® dsDNA System (Promega, Madison, USA), respectively.

Illumina libraries were prepared with the Illumina DNA prep kit (Illumina, San Diego, USA) and Nextera DNA CD Indexes (Illumina, San Diego, USA), using approximately 400 ng of input DNA. The libraries were normalized using a bead-based procedure and pooled together at equal volume. The pooled library was denatured and sequenced using Miseq reagent version 2 (Illumina, Inc. San Diego, CA) on an Illumina Miseq platform with  $2 \times 150$  paired-end chemistry.

## Genome assembly and quality assessment

Illumina fastQ reads assembly was conducted using Bactopia v2.2.0 [32]. Shovill v1.1.0 (<https://github.com/tseemann/shovill>) was used to perform the genome assembly, whereas quast v5.0.2 [33] was used to evaluate the genomic assembly. The resulting genome comprised 27 contigs with an overall coverage of  $70\times$ .

## Bioinformatic analysis

**Detection of transferable antimicrobial and heavy metal resistance genes.** AMRFinderPlus v3.11.20 was used for detection of antimicrobial and antimicrobial resistance genes.

This software uses the curated Reference Gene Database of the National Center for Biotechnology Information.

**Detection of chromosomal point mutations.** Mutations in chromosomal genes involved in the development of antimicrobial resistance in *gyrA*, *gyrB*, *parC*, *parE* (quinolones), *rpoB* (rifampicins), *rplD*, *rplV* (macrolides), *rpsE*, *rpsG*, (aminoglycosides), *mrcA*, *mrcB*, *mrDA*, *ftsI* ( $\beta$ -lactams), *lpxA*, *lpxB*, *lpxC*, *lpxD*, *lpxL*, *lpxM*, *lpsB* and *pmrC* genes (polymyxins) as well as in 16s rRNA (resistance to tetracyclines) and 23s rRNA (resistance to macrolides and anfenicols) were analyzed comparing the obtained sequences with both the *A. radioresistens* GenBank reference genome LH6 (Accession number CP030031.1) and the type strain DSM 6976 (Accession number AP019740.1). Only amino acid differences (or base differences for 16s rRNA and 23s rRNA) shared with both reference genomes were considered.

**Detection of virulence factors.** The automatic pipeline VF analyzer (<http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi>) was employed for analysis of virulence factor-encoding genes, using raw FASTA sequence as input.

**Phylogenomic comparison and pangenome.** A total of 153 *A. radioresistens* genomes and corresponding metadata were downloaded using the NCBI datasets command-line tools on January 10, 2024, considering both draft genomes and complete assemblies. Genome metrics were obtained with Quast v. 5.2.0 [34], discarding 53 genomes due to quality issues. To ensure reducing bias from different annotation algorithms, the 100 remaining genomes were annotated using Prokka v. 1.14.6. [35]. The pangenome was calculated using Roary v. 3.13 and Snp-sites v. 2.5.1 was employed to extract SNPs from the multi-alignment [36, 37]. The phylogenomic tree was then constructed using IQ-TREE v. 2.06. with automatic model selection and ultrafast Bootstrap for 1,000 replicates [38–40]. Tree visualization and pangenome representation were obtained with FigTree v. 1.4.4. and Phandango respectively [41, 42]. The definition criteria for Core and Soft genome was the presence in the 99% and 95% of analyzed genomes.

Afterwards, the closest genome and the closest confirmed human pathogenic clinical isolates were selected to calculate the average nucleotide identity (ANI), using the OrthoANIu algorithm [43].

## Multilocus sequence typing detection

In the absence of a specific sequence typing (ST) scheme for *A. radioresistens*, the ST pattern was established following the *A. baumannii* Pasteur scheme (<https://pubmlst.org/organisms/acinetobacter-baumannii>), which also considers *Acinetobacter* species other than *A. baumannii*.

**Lipooligosaccharide outer core and capsule.** The presence and type of genes involved in lipooligosaccharide outer core (OCL) and capsule synthesis was determined using Kaptive (<https://kaptive-web.erc.monash.edu/>).



## Data availability statement

Genomic data are recorded under Genome assembly and Sequencing project ASM3404387v1 and JAXCJJ00000000.1 respectively, with the accession code: GCA\_034043875.1; Sequence Read Archive accession for the fastQ reads is SRR26939120. Access to the Illumina fastQ reads and the assembled genome is available through the NCBI BioProject PRJNA1044581.

## Ethics approval

This study was approved by the Institutional Ethics Committee in Research with Animals and Biodiversity (Code N° 076-CIEI-AB-CIENTÍFICA-2020) of the Universidad Científica del Sur. Permits to work on Guañape Norte island and swabbing the birds were issued by SERNANP (RJ-012-2019-SERNANP-RNSIIPG). Likewise, permits to use guano island installations were issued by Agrorural (Oficio 0249-2019-MINAGRI-DVDIARAGRORURAL-DAB).

## RESULTS

In the course of the study, 23 non-duplicate microorganisms were recovered from 15 Humboldt penguins sampled, including *A. radioresistens* (12 isolates), *K. oxytoca* (4 isolates), *Pseudomonas* spp. (2 isolates), *Pseudomonas putida* (2 isolates), *Pseudomonas aeruginosa* (1 isolate), *Morganella morganii* (1 isolate) and *Pantoea* spp. (1 isolate). In all cases the identification at the species level was established by MALDI-TOF MS.

The 12 non-duplicate *A. radioresistens* were included in further studies. The *A. radioresistens* isolates presented the *bla*<sub>OXA-23</sub> gene and showed high levels of antibiotic susceptibility, with only 8 isolates showing intermediate resistance to cefotaxime. Interestingly, the clonal analysis,

performed by either PFGE or FTIR spectroscopy, showed that all the isolates were genetically related and could be included within the same clonal cluster (Fig. 1). Subsequently, a representative isolate, designated as *A. radioresistens* 4A, was further selected for a full characterization through whole genome sequencing (WGS) analysis (Fig. 2).

WGS confirmed the presence of *bla*<sub>OXA-23G</sub>, which was identified as the *bla*<sub>OXA-23</sub> gene. Additionally, despite the lack of resistance to carbapenems, the presence of a chromosomal putative subclass B3 metallo-beta-lactamase showing 100% identity with that of the *A. radioresistens* strain FDAARGOS\_731 (GenBank access: CP059684.1), was also detected. Alterations in chromosomal genes either directly encoding antibiotic targets or involved in metabolic processes that may lead to antibiotic-susceptible or antibiotic-resistant phenotypes were sought, but while a series of point mutations were detected, no apparent effect on antibiotic resistance was found, and they were thereby classified as polymorphism (Table 1). Meanwhile, no gene involved in resistance to heavy metals was detected. On the other side, the analysis also showed the presence of several virulence factors, including genes related to OCL, high-affinity iron-chelating compound receptors, phospholipases, among others (Table 2), and allowed assigning the representative isolate to ST1972. Regarding OCL, the analysis detected eight genes close related to OCL3 (identities varying from 94.3% to 100%), with an overall identity degree of 91.4%. Of note, the homologous gene to *gtr*<sub>OCL</sub> was not identified (Table 2). Regarding capsule, the analysis only detected the presence of 8 out of 20 genes involved in its synthesis, and thereby was not further examined.

The obtained phylogenomic tree showed that, in general, *A. radioresistens* can be divided in two major clades, and that confirmed human pathogenic *A. radioresistens* are not restricted to one clade only. The genome from this study was more related to GCA\_023130635.1 (ANI 99.96%), which is a

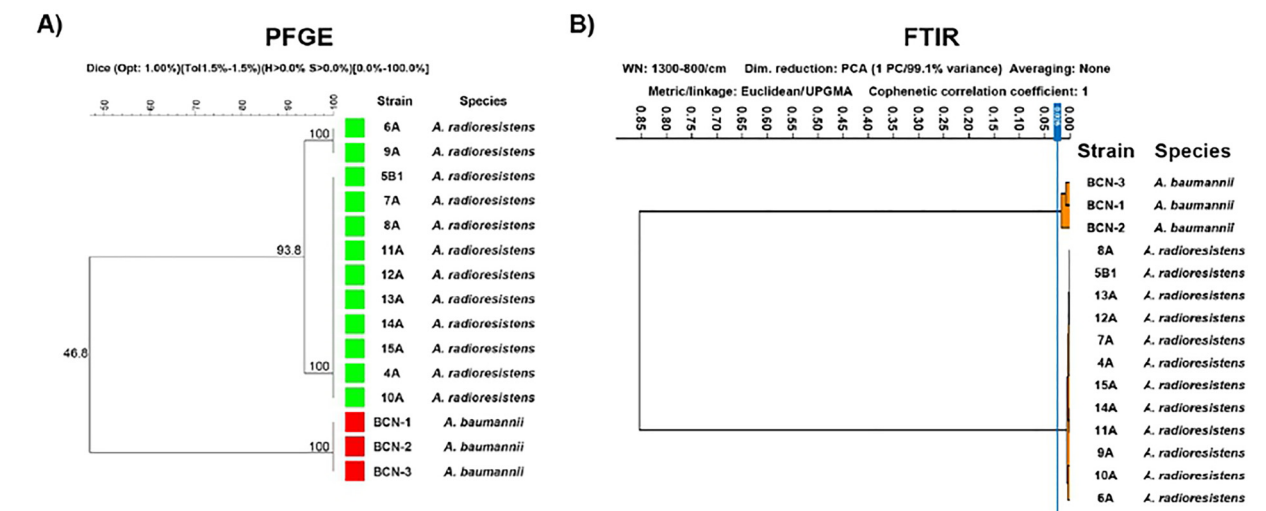
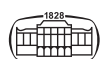


Fig. 1. (A) PFGE and (B) FTIR dendrograms depicting the clonal relatedness of *Acinetobacter radioresistens* isolates recovered from Humboldt penguins and three *Acinetobacter baumannii* isolates included as an outgroup



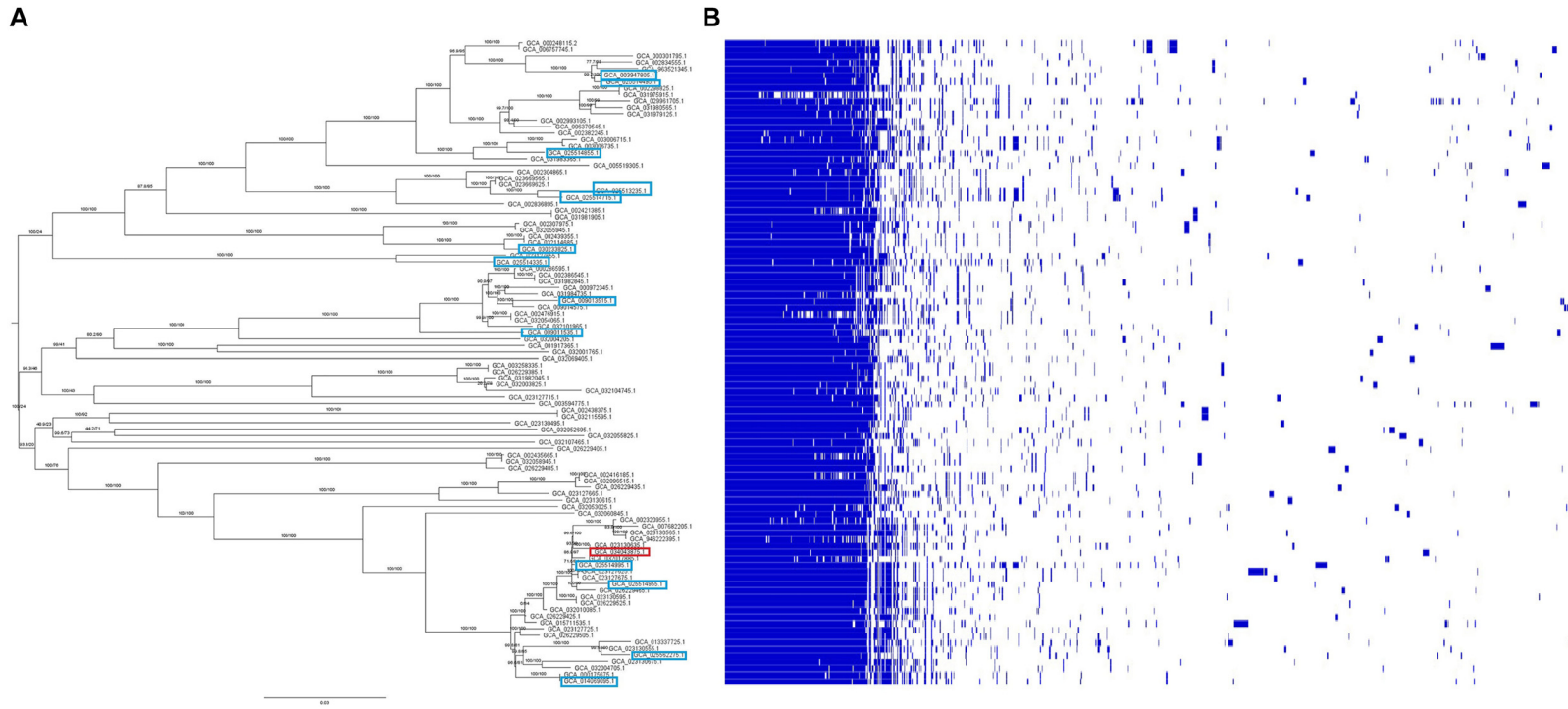


Fig. 2. Phylogenomic tree and pangenome representation of the analyzed genomes

A: Phylogenomic tree.

The genome from this study is shown within the red box, whilst Genomes in light blue boxes are confirmed clinical isolates/pathogenic *A. radioresistens*.

B: Pangenome of *Acinetobacter radioresistens*.

The core genome is considered when the genes are present in 99% of the isolates.

Table 1. Chromosomal point mutations in antibiotic resistance-related genes

Genes	Point Mutations <sup>1</sup>	Resistance <sup>2</sup>
<i>gyrA</i>	A510V, V529I, T684M, E981Q	Quinolones
<i>gyrB</i>	R219C	Quinolones
<i>parC</i>	E356G, H395R	Quinolones
16S rRNA	T1449C (DNA)	Tetracyclines
<i>mrcB</i> (PBP1b)	D85N, S134A, E336D	β-lactams
<i>mrda</i> (PBP2)	A402T, T660M	β-lactams
<i>ftsI</i> (PBP3)	P15S, N156K	β-lactams
<i>lpxA</i>	I31V	Polymyxins
<i>lpxB</i>	P161S, P343A	Polymyxins
<i>lpxC</i>	K8R	Polymyxins
<i>lpsB</i>	S71P, K74R, I95T, A125E, Q370R	Polymyxins
<i>pmrB</i>	A55T, R260K	Polymyxins

No alterations were found in the remaining genes analyzed.

<sup>1</sup> Only reported differences common to both strain LH6 (GenBank: CP030031.1) and strain DSM 6976 (GenBank: AP019740.1).

<sup>2</sup> Antibiotic resistance which has been related to the presence of specific alterations in these genes.

Table 2. Virulence factors

Genes	Virulence factor
orfs 2549-2555; orfs 2561-2569	Lipooligosaccharide outer core locus
<i>plcN</i>	Phospholipase C
<i>plD-1</i>	Phospholipase D
<i>plD-2</i>	Phospholipase D
<i>fepA</i>	Siderophore receptor
<i>bfr-1</i>	bacterioferritin
<i>bfr-2</i>	bacterioferritin
<i>katA</i>	Catalase
<i>katB</i>	Catalase
<i>katD</i>	Catalase
<i>sodB</i>	Superoxide dismutase
<i>sodC</i>	Superoxide dismutase

Open reading frames (Orfs) indicated following numeration of the draft genome of isolate 4A (GenBank access JAXCJJ000000000.1).

German isolate from a human urine sample collected in 2013. Moreover, the other two closest pathogenic isolates GCA\_025514995.1 and GCA\_025514955.1 (ANI 99.82% and 99.81% respectively) were collected in 2019 from a Hospital in the United States of America. The pangenome showed a total of 14,209 genes, with only “core” and “soft core” genes being 1,108 and 901 respectively. Many of the accessory genes classified as hypothetical proteins, plasmid genes, insertion sequences and transposases.

## DISCUSSION

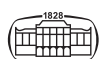
*A. radioresistens* is an environmental microorganism [6], which has also been involved in opportunistic infections

[8, 9, 17]. While the isolation of *A. radioresistens* in wild animals has previously been described, with descriptions in frogs or yellow-legged gulls [18, 20], in the present study the common presence of *A. radioresistens* as an apparent commensal inhabitant of the gut microbiota of Humboldt penguins is reported for the first time. Of note, neither selective media for detection of *Acinetobacter* spp., nor culture-independent techniques for the detection of *Acinetobacter* species were used. Nevertheless, while most studies focused on the direct or indirect detection of microorganisms in wild or captive Humboldt penguins have been addressed to pathogenic issues [44–46], in one study describing the gut microbiota of captive Humboldt penguins, the presence of *Acinetobacter* spp. ranked among the taxa with the highest relative abundance [47].

In agreement with a low anthropic pressure and feeding habits, the present isolates were susceptible to all the antimicrobial agents tested, except for 8 cefotaxime intermediate isolates. This finding corroborates similar results regarding the recovery of *Acinetobacter* spp. and *Pseudomonas* spp. from cloacal swabs from Peruvian boobies inhabiting the same island [3]. Of note, *Escherichia coli* recovered from the above-mentioned Peruvian boobies presented resistance to several antimicrobial agents [3]. It is worth mentioning that clinical reports of *A. radioresistens* often shows a similar scenario of antibiotic susceptibility [8–10]. Eight isolates displayed intermediate susceptibility to cefotaxime, with no specific mechanisms being identified. Thereby this phenomenon might be related to differences in the expression levels of chromosomal efflux pumps, with this role confirmed in other species of the genera [48].

According to the results of two different techniques, all the isolates belonged to the same clonal cluster, likely related to the density of the Humboldt penguin colony which might favor microorganism sharing. This finding showing than in densely populated bird colonies, microorganisms either infecting birds or inhabiting their microbiotas may easily be dispersed and shared. Of note, an unidentified fully antibiotic-susceptible *Acinetobacter* spp. was recovered from a Peruvian booby during the same sampling [3].

Despite the above-mentioned antibiotic susceptibility, the isolates presented two carbapenemases and several antibiotic-target alterations. OXA-23 is intrinsic of this microorganism, but its standard low level of expression does not allow the development of resistance to carbapenems [11]. Nevertheless, the mobilization of OXA-23, due to insertion sequences, to other members of the genus (such as *A. baumannii*) results in high levels of resistance to carbapenems due to increasing levels of expression [11]. On the other hand, there is limited data regarding the putative subclass B3 metallo-beta-lactamase that also appears to be intrinsic of *A. radioresistens* [49], but a similar scenario of low/null expression in the absence of deregulation seems plausible. Of note, while a series of point mutations in several genes commonly known to be associated with the development of antibiotic resistance were identified, the overall antibiotic susceptibility levels strongly suggest the presence of nucleotide polymorphisms with no impact on



the development of antibiotic resistance. Finally, no specific reason was found to explain the isolates intermediate to cefotaxime.

The presence of virulence factors, including siderophore acquisition system, shows the potential of these isolates to produce infections in both humans and animals. Despite 153 *A. radioresistens* genomes are present in GenBank, no specific data about the OCL diversity in this specie has been found. OCL3 it has been described as present in international clones (IC) of high risk of *A. baumannii* such as IC1 or IC2 [50]. Of note, potential health issues related to the presence of *A. radioresistens* were not observed in the penguins sampled or in posterior reports from the Humboldt penguin colony. These findings suggest that *A. radioresistens* is a commensal, or at least innocuous resident, in the gut microbiota of Humboldt penguins.

A search in <https://pubmlst.org/organisms/acinetobacter-baumannii> showed that ST1972 has only been reported twice: isolates FDAArgos\_731 (GenBank access: CP059684.1) and MGYG-HGUT-01344 (GenBank access: CABKOV01000001-CABKOV010000018). In both cases *A. radioresistens* were from a human origin (skin and gut). This finding suggests ease of interspecies spread from human to animals or vice versa.

While there are previous genomic comparison studies of *Acinetobacter* spp. which naturally include *A. radioresistens* [51], to the best of our knowledge, this study includes the first pangenomic comparison amongst genomes of *A. radioresistens* only. Of note, confirmed human pathogenic isolates were non-grouped, thereby no specific association of them with present isolate was done. Regarding isolates belonging to ST1972 both were excluded for comparisons due to genome quality issues.

The main limitation of this study is the time lapse from samples acquisition to laboratory culture, which was related to the nature of the sampling. In addition, the same delay in sample processing might affect bacterial viability of several microorganisms, resulting facilitating the isolation of *A. radioresistens*.

In summary, the present study highlights the clonal spread of *A. radioresistens* in a Humboldt penguin colony. Further studies in other geographical areas are needed to establish the commonness of this microorganism as an inhabitant of the Humboldt penguin gut, to determine if it belongs to the normal gut microbiota or if the present results show a local phenomenon.

**Funding sources:** This study was supported by the *Fondo Nacional de Desarrollo Científico, Tecnológico y de Innovación Tecnológica* (FONDECYT - Perú) and Universidad Científica del Sur within the “*Proyecto de Mejoramiento y Ampliación de los Servicios del Sistema Nacional de Ciencia, Tecnología e Innovación Tecnológica*” [contract: 08-2019-FONDECYT-BM-INC-INV], and by the *Programa Nacional de Pesca y Acuicultura* (PNIPA-Peru) and Universidad Científica del Sur under the project “*Aves Marinas como Centinelas del Mar: Uso de Video-cámaras y GPS para monitorear actividades pesqueras ilícitas*”. This study was also supported by the I+D+i grant PID2021-127402OB-I00,

funded by MCIN/AEI/10.13039/501100011033, co-financed by the European Development Regional Fund “A way to achieve Europe” and grant 2017 SGR 0809 from the *Departament d’Universitats, Recerca i Societat de la Informació*, of the Generalitat de Catalunya. We also acknowledge support from the Spanish Ministry of Science, Innovation and Universities through the “*Centro de Excelencia Severo Ochoa 2019–2023*” Program (CEX2018-000806-S), and support from the *Generalitat de Catalunya* through the CERCA Program”. J.V. is the recipient of a Margarita Salas grant (*Universitat Autònoma de Barcelona*, 2021) from the Spanish Ministerio de Universidades.

**Authors’ contributions:** I.R, C.Z., M.J.P. and J.R. conceived and designed the study; C.I.-L., D.D.GDC. and C.Z. performed the sampling. K.E., M.P., A.K.C., L.M., B.Y. and R.O. performed microbiological analyses. L.A.P-K and J.V. performed genomic analysis. J.R. wrote the initial version. All authors have read, revised, and agreed to the final version of the manuscript.

**Conflict of interest:** The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

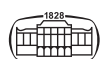
We thank Donna Pringle for idiomatic correction.

## REFERENCES

- Levy-Blitchtein S, Roca I, Plasencia-Rebata S, Vicente-Taboada W, Velázquez-Pomar J, Muñoz L, et al. Emergence and spread of carbapenem-resistant *Acinetobacter baumannii* international clones II and III in Lima, Peru. *Emerg Microbes Infect.* 2018;7:119. <https://doi.org/10.1038/s41426-018-0127-9>.
- Wisplinghoff H, Paulus T, Lugenheim M, Stefanik D, Higgins PG, Edmond MB, et al. Nosocomial bloodstream infections due to *Acinetobacter baumannii*, *Acinetobacter pittii* and *Acinetobacter nosocomialis* in the United States. *J Infect.* 2012;64:282–90. <https://doi.org/10.1016/j.jinf.2011.12.008>.
- Espinoza K, Zavalaga C, Irigoien-Lovera C, Gonzales-DelCarpio DD, Díaz-Santibañez I, Pons MJ, et al. Antimicrobial resistance levels among Gram-negative bacteria from Peruvian Boobies (*Sula variegata*) in Northern Peru. *J Wildl Dis.* 2021;57:722–5. <https://doi.org/10.7589/JWD-D-21-00003>.
- Maboni G, Seguel M, Lorton A, Sanchez S. Antimicrobial resistance patterns of *Acinetobacter* spp. of animal origin reveal high rate of multidrug resistance. *Vet Microbiol.* 2020;245:108702. <https://doi.org/10.1016/j.vetmic.2020.108702>.
- Marí-Almirall M, Cosgaya C, Pons MJ, Nemeč A, Ochoa TJ, Ruiz J, et al. Pathogenic *Acinetobacter* species including the novel *Acinetobacter dijkschoorniae* recovered from market meat in Peru. *Int J Food Microbiol.* 2019;305:108248. <https://doi.org/10.1016/j.ijfoodmicro.2019.108248>.



6. Nishimura Y, Ino T, Iizuka H. *Acinetobacter radioresistens* sp. nov. isolated from cotton and soil. *Int J Syst Bacteriol* 1988;38: 209–11.
7. Rafei R, Hamze M, Pailhoriès H, Eveillard M, Marsollier L, Joly-Guillou ML, et al. Extrahuman epidemiology of *Acinetobacter baumannii* in Lebanon. *Appl Environ Microbiol*. 2015;81:2359–67. <https://doi.org/10.1128/AEM.03824-14>.
8. Visca P, Petrucca A, De Mori P, Festa A, Boumis E, Antinori A, et al. Community-acquired *Acinetobacter radioresistens* bacteremia in an HIV-positive patient. *Emerg Infect Dis*. 2001;7:1032–5. <https://doi.org/10.3201/eid0706.010621>.
9. Wang T, Costa V, Jenkins SG, Hartman BJ, Westblade LF. *Acinetobacter radioresistens* infection with bacteremia and pneumonia. *IDCases*. 2019;15:e00495. <https://doi.org/10.1016/j.idcr.2019.e00495>.
10. Bello-López E, Rocha-Gracia RdC, Castro-Jaimes S, Cevallos MÁ, Vargas-Cruz M, Verdugo-Yocupicio R, et al. Antibiotic resistance mechanisms in *Acinetobacter* spp. strains isolated from patients in a paediatric hospital in Mexico. *J Glob Antimicrob Resist*. 2020;23: 120–9. <https://doi.org/10.1016/j.jgar.2020.08.014>.
11. Barrientos-Yong RS, Hinojosa-Salas BA, Salas-Ponce PG, Angles E, Ruiz J, Pons MJ. High rates of extensively drug-resistant *Acinetobacter baumannii* in a Peruvian hospital 2013–2019. *Trop Doct*. 2023;53:248–55. <https://doi.org/10.1177/00494755221142939>.
12. Mosqueda N, Espinal P, Cosgaya C, Viota S, Plasencia V, Álvarez-Lerma F, et al. Globally expanding carbapenemase finally appears in Spain: nosocomial outbreak of *Acinetobacter baumannii* producing plasmid-encoded OXA-23 in Barcelona, Spain. *Antimicrob Agents Chemother*. 2013;57:5155–7. <https://doi.org/10.1128/AAC.01486-13>.
13. Yin L, Wu N, Yan G, Lu L, Qian H, Yang W, et al. Carbapenem-resistant gram-negative bacterial prevention practice in nosocomial infection and molecular epidemiological characteristics in a pediatric intensive care unit. *Heliyon*. 2023;9:e18969. <https://doi.org/10.1016/j.heliyon.2023.e18969>.
14. Poirel L, Figueiredo S, Cattoir V, Carattoli A, Nordmann P. *Acinetobacter radioresistens* as a silent source of carbapenem resistance for *Acinetobacter* spp. *Antimicrob Agents Chemother*. 2008;52: 1252–6. <https://doi.org/10.1128/AAC.01304-07>.
15. Noel HR, Petrey JR, Palmer LD. Mobile genetic elements in *Acinetobacter* antibiotic-resistance acquisition and dissemination. *Ann N Y Acad Sci*. 2022;1518:166–82. <https://doi.org/10.1111/nyas.14918>.
16. Al Atrouni A, Joly-Guillou ML, Hamze M, Kempf M. Reservoirs of non-baumannii *Acinetobacter* species. *Front Microbiol*. 2016;7:49. <https://doi.org/10.3389/fmicb.2016.00049>.
17. Opazo-Capurro A, Higgins PG, Wille J, Seifert H, Cigarroa C, González-Muñoz P, et al. Genetic features of Antarctic *Acinetobacter radioresistens* strain A154 harboring multiple antibiotic-resistance genes. *Front Cell Infect Microbiol*. 2019;9:328. <https://doi.org/10.3389/fcimb.2019.00328>.
18. Ngaiganam EP, Rolain JM, Diene SM. Detection of a new variant of OXA-23 carbapenemase in *Acinetobacter radioresistens* isolates from urban animals in Marseille, France. *J Glob Antimicrob Resist*. 2019;16:178–80. <https://doi.org/10.1016/j.jgar.2019.01.021>.
19. Unger F, Eisenberg T, Prenger-Berninghoff E, Leidner U, Semmler T, Ewers C. Imported pet reptiles and their “blind passengers”-in-depth characterization of 80 *Acinetobacter* species isolates. *Microorganisms*. 2022;10:893. <https://doi.org/10.3390/microorganisms10050893>.
20. Cevallos MA, Basanta MD, Bello-López E, Escobedo-Muñoz AS, González-Serrano FM, Nemeč A, et al. Genomic characterization of antifungal *Acinetobacter* bacteria isolated from the skin of the frogs *Agalychnis callidryas* and *Craugastor fitzingeri*. *FEMS Microbiol Ecol* 2022;98:fiac126. <https://doi.org/10.1093/femsec/fiac126>.
21. Agrorural. Reporte mensual de conservación en islas y puntas guaneras. 2023. <https://www.agrorural.gob.pe/wp-content/uploads/2023/02/REPORTE-MENSUAL-CONSERVACION-WEB-1.pdf>. Accessed on December 12, 2023.
22. Dantas GPM, Oliveira LR, Santos AM, Flores MD, de Melo DR, Simeone A, et al. Uncovering population structure in the Humboldt penguin (*Spheniscus humboldti*) along the Pacific coast at South America. *Plos One*. 2019;14:e0215293. <https://doi.org/10.1371/journal.pone.0215293>.
23. Sallaberry-Pincheira N, González-Acuña D, Padilla P, Dantas GPM, Luna-Jorquera G, Frere E, et al. Contrasting patterns of selection between MHC I and II across populations of Humboldt and Magellanic penguins. *Ecol Evol*. 2016; 6: 7498–510. <https://doi.org/10.1002/ece3.2502>.
24. Espinal PA, Macia MD, Roca I, Gato E, Ruiz E, Fernández-Cuenca F, et al. First report of an OXA-23 carbapenemase-producing *Acinetobacter baumannii* clinical isolate related to Tn2006 in Spain. *Antimicrob Agents Chemother*. 2013;57:589–91. <https://doi.org/10.1128/aac.01157-12>.
25. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Supplement M100 - S33. Wayne: CLSI; 2023.
26. Naomi-Matsuoka A, Vargas M, Ymaña B, Soza G, Pons MJ. Colistin resistance in multidrug-resistant *Klebsiella pneumoniae* strains at a perinatal maternal institute in Lima, Peru, 2015–2018. *Rev Peru Med Exp Salud Publica*. 2020;37:716–20. <https://doi.org/10.17843/rpmesp.2020.374.5422>.
27. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters version 13. 2023. [https://www.eucast.org/clinical\\_breakpoints](https://www.eucast.org/clinical_breakpoints). Accessed on December 12, 2023.
28. Food and Drug Administration (FDA). 2023. <https://www.fda.gov/drugs/development-resources/tigecycline-injection-products>. Accessed on December 12, 2023.
29. Jones RN, Ferraro MJ, Reller LB, Schreckenberger PC, Swenson JM, Sader HS. Multicenter studies of tigecycline disk diffusion susceptibility results for *Acinetobacter* spp. *J Clin Microbiol*. 2007;45: 227–30. <https://doi.org/10.1128/JCM.01588-06>.
30. Uwingabiye J, Lemnouer A, Roca I, Alouane T, Frikh M, Belefquih B, et al. Clonal diversity and detection of carbapenem resistance encoding genes among multidrug-resistant *Acinetobacter baumannii* isolates recovered from patients and environment in two intensive care units in a Moroccan hospital. *Antimicrob Resist Infect Control*. 2017;6:99. <https://doi.org/10.1186/s13756-017-0262-4>.
31. Durmaz R, Otlu B, Koksall F, Hosoglu S, Ozturk R, Ersoy Y, et al. The optimization of a rapid pulsed-field gel electrophoresis protocol for the typing of *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella* spp. *Jpn J Infect Dis*. 2009;62:372–7.





32. Petit RA 3rd, Read TD. Bactopia: a flexible pipeline for complete analysis of bacterial genomes. *mSystems*. 2020;5:e00190–20. <https://doi.org/10.1128/mSystems.00190-20>.
33. Mikheenko A, Pribelski A, Saveliev V, Antipov D, Gurevich A. Versatile genome assembly evaluation with QUASt-LG. *Bioinformatics*. 2018;34:i142–50. <https://doi.org/10.1093/bioinformatics/bty266>.
34. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUASt: quality assessment tool for genome assemblies. *Bioinformatics*. 2013;29:1072–5. <https://doi.org/10.1093/bioinformatics/btt086>.
35. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics*. 2014;30:2068–9. <https://doi.org/10.1093/bioinformatics/btu153>.
36. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, et al. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics*. 2015;31:3691–3. <https://doi.org/10.1093/bioinformatics/btv421>.
37. Page AJ, Taylor B, Delaney AJ, Soares J, Seemann T, Keane JA, et al. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb Genom*. 2016;2:e000056. <https://doi.org/10.1099/mgen.0.000056>.
38. Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. UFBoot2: improving the ultrafast bootstrap approximation. *Mol Biol Evol*. 2018;35:518–22. <https://doi.org/10.1093/molbev/msx281>.
39. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermini LS. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods*. 2017;14:587–9. <https://doi.org/10.1038/nmeth.4285>.
40. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 2015;32:268–74. <https://doi.org/10.1093/molbev/msu300>.
41. Hadfield J, Croucher NJ, Goater RJ, Abudahab K, Aanensen DM, Harris SR. Phandango: an interactive viewer for bacterial population genomics. *Bioinformatics*. 2018;34(2):292–3. <https://doi.org/10.1093/bioinformatics/btx610>.
42. Rambaut A. FigTree v1.3.1. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh; 2010. Available at: <http://tree.bio.ed.ac.uk/software/figtree/>.
43. Yoon SH, Ha SM, Lim J, Kwon S, Chun J. A large-scale evaluation of algorithms to calculate average nucleotide identity. *Antonie Van Leeuwenhoek*. 2017;110(10):1281–6. <https://doi.org/10.1007/s10482-017-0844-4>.
44. Bralet T, Risco-Castillo V, Valsecchi I, Laidebeure S, Sailler A, Lécru A, et al. Aspergillosis in a colony of Humboldt penguins (*Spheniscus humboldti*) in a French zoological park: evaluation of environmental exposure. *Vet Res Commun*. in press. <https://doi.org/10.1007/s11259-023-10220-z>.
45. Smith KM, Karesh WB, Majluf P, Paredes R, Zavalaga C, Reul AH, et al. Health evaluation of free-ranging Humboldt penguins (*Spheniscus humboldti*) in Peru. *Avian Dis*. 2008;52:130–5. <https://doi.org/10.1637/8265-071007-Reg>.
46. Widmer D, Ziemssen E, Schade B, Kappe E, Schmitt F, Kempf H, et al. *Pseudomonas aeruginosa* infection in a group of captive Humboldt penguins (*Spheniscus humboldti*). *J Avian Med Surg*. 2016;30:187–95. <https://doi.org/10.1647/2015-107>.
47. Thorel M, Mateos-Hernandez L, Mulet B, Azzouni MN, Hodžić A, Gaillot H, et al. Assessment of the safety and efficacy of an oral probiotic-based vaccine against *Aspergillus* infection in captive-bred Humboldt Penguins (*Spheniscus humboldti*). *Front Immunol*. 2022;13:897223. <https://doi.org/10.3389/fimmu.2022.897223>.
48. Kim CM, Park G, Ko YJ, Kang SH, Jang SJ. Relationships between relative expression of RND efflux pump genes, H33342 efflux activity, biofilm-forming activity, and antimicrobial resistance in *Acinetobacter baumannii* clinical isolates. *Jpn J Infect Dis*. 2021;74:499–506. <https://doi.org/10.7883/yoken.JJID.2020.765>.
49. Crippen CS, Huynh S, Miller WG, Parker CT, Szymanski CM. Complete genome sequence of *Acinetobacter radioresistens* strain LH6, a multidrug-resistant bacteriophage-propagating strain. *Microbiol Resour Announc*. 2018;7:e00929–18. <https://doi.org/10.1128/MRA.00929-18>.
50. Kenyon JJ, Nigro SJ, Hall RM. Variation in the OC locus of *Acinetobacter baumannii* genomes predicts extensive structural diversity in the lipooligosaccharide. *PLoS One*. 2014;9:e107833. <https://doi.org/10.1371/journal.pone.0107833>.
51. Saffarian A, Touchon M, Mulet C, Tournebize R, Passet V, Brisse S, et al. Comparative genomic analysis of *Acinetobacter* strains isolated from murine colonic crypts. *BMC Genomics*. 2017;18:525. <https://doi.org/10.1186/s12864-017-3925-x>.

**Open Access statement.** This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<https://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium for non-commercial purposes, provided the original author and source are credited, a link to the CC License is provided, and changes – if any – are indicated.

