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Published in:
Access Microbiology

DOI:
10.1099/acmi.0.000694.v3

First published: 09/11/2023

Document Version
Publisher's PDF, also known as Version of record

Link to publication

Citation for pulished version (APA):
Negus, D., Foster, G., & Hoyles, L. (2023). Lelliottia amnigena recovered from the lung of a harbour porpoise, and comparative analyses with Lelliottia spp. *Access Microbiology*, *5*(11). Advance online publication. https://doi.org/10.1099/acmi.0.000694.v3

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SHORT COMMUNICATION
Negus *et al.*, *Access Microbiology* 2023;5:000694.v3
DOI 10.1099/acmi.0.000694.v3

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Lelliottia amnigena recovered from the lung of a harbour porpoise, and comparative analyses with *Lelliottia* spp.

David Negus[1], Geoffrey Foster[2] and Lesley Hoyles[1,*]

Abstract

Strain M1325/93/1 (herein referred to by our laboratory identifier, GFKo1) of *Lelliottia amnigena* was isolated from the lung of a harbour porpoise in 1993. The genome sequence and antimicrobial resistance profile (genomic, phenotypic) of the strain were generated, with the genomic data compared with those from closely related bacteria. We demonstrate that the recently described chromosomally encoded AmpC β-lactamase bla_{LAQ} is a core gene of *L. amnigena*, and suggest that new variants of this class of lactamase are encoded by other members of the genus *Lelliottia*. Although presence of bla_{LAQ} is ubiquitous across the currently sequenced members of *L. amnigena*, we highlight that strain GFKo1 is sensitive to ampicillin and cephalosporins. These data suggest that bla_{LAQ} may act as a useful genetic marker for identification of *L. amnigena* strains, but its presence may not correlate with expected phenotypic resistances. Further studies are required to determine the regulatory mechanisms of bla_{LAQ} in *L. amnigena*.

DATA SUMMARY

Supplementary material associated with this article is available from figshare: https://figshare.com/projects/Lelliottia_amnigena_characterization/174210 [1–4]. The whole-genome sequence data generated for this study are available from BioProject PRJNA979992.

INTRODUCTION

Lelliottia spp. are Gram-negative, facultatively anaerobic bacteria of the family *Enterobacteriaceae*. The genus *Lelliottia* was created to accommodate species distinct from *Enterobacter sensu lato* based on *gyrB*, *rpoB*, *infB* and *atpD* gene sequence analyses, and comprises four species with validly published names (*Lelliottia amnigena*, *Lelliottia aquatilis*, *Lelliottia jeotgali* and *Lelliottia nimipressuralis*) and one with a non-valid name ('*Lelliottia steviae*') [5–8]. *Lelliottia aquatilis* represents a later heterotypic synonym of *L. jeotgali*, based on average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization analyses [9].

Lelliottia spp. have been associated with the commensal microbiota of flies and the Asian tiger mosquito [10, 11], and isolated from fresh and wastewater, soil, plants, air samples and fish [6, 7, 12–19]. Interest in *L. amnigena* is increasing as this bacterium has been associated with soft rot of economically important plant crops such as onion and potato [20]. Only rarely have *L. amnigena* and *L. nimipressuralis* been associated with opportunistic disease in humans [21–24]. There are few reports in the literature of the carriage of antimicrobial resistance (AMR) genes by *Lelliottia* spp., though a new chromosomally encoded AmpC β-lactamase, $bla_{LAQ\text{-}1}$, conferring resistance to ampicillin and several cephalosporins was recently described for an *L. amnigena* strain isolated from animal farm sewage in PR China [25, 26].

As part of a study of veterinary isolates thought to belong to the *Klebsiella oxytoca* complex [27], we identified several atypical strains that were shown by *rpoB* gene sequence analysis to represent a range of different *Enterobacteriaceae* [28]. Here, we report

Received 28 August 2023; Accepted 20 October 2023; Published 09 November 2023
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Keywords: AmpC; antimicrobial resistance; bla_{LAQ}; *Huaxiibacter*; marine; veterinary microbiology.
Abbreviations: AMR, antimicrobial resistance; ANI, average nucleotide identity; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MAG, metagenome-assembled genome; oANI, orthologous average nucleotide identity; rMLST, ribosomal multilocus sequence typing.
The whole-genome sequence data generated for this study are available from BioProject PRJNA979992.
Four supplementary figures and three supplementary tables are available with the online version of this article.
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on one such strain recovered from the lung of a harbour porpoise (*Phocoena phocoena*). Using genome sequence data and comparative analyses, we demonstrate that this is a strain of *L. amnigena* and compare its AMR gene profile with those of publicly available sequence data for the species.

METHODS

Isolation and phenotypic characterization of strain

Strain M1325/93/1 (herein referred to by our laboratory identifier, GFKo1) was isolated on Columbia sheep blood agar (Oxoid, Basingstoke, UK) from the lung of a harbour porpoise that was found stranded at Buckie on the southern coastline of the Moray Firth, north-east Scotland in June 1993. Tentative identification and biochemical characterization of the strain were performed using the API 20E (bioMérieux) strip according to the manufacturer's instructions under aerobic conditions at 37°C. The isolate was also identified by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS) using the Bruker Microflex LT/SH MALDI-TOF MS Biotyper. Antimicrobial sensitivity testing was performed by disc diffusion assays following guidelines from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) v 13.1 for *Enterobacterales. Escherichia coli* ATCC 25922 was used as the reference strain for quality control purposes. All antibiotics were purchased from Oxoid, UK.

DNA extraction and sequencing

DNA was extracted from an overnight culture (aerobic, 37°C) of strain GFKo1 grown in nutrient broth (Oxoid) using the Qiagen DNeasy Blood and Tissue kit (Qiagen). Extracted DNA was adjusted to a concentration of 0.2 ng μl^{-1} and treated using the Nextera XT DNA library preparation kit (Illumina) to produce fragments of approximately 500 bp. Fragmented and indexed samples were run on the sequencer using the MiSeq Reagent kit v2 (Illumina; 250 bp paired-end reads) following Illumina's recommended denaturation and loading procedures.

Genome assembly and gene annotation

Raw sequence data were checked using fastqc v0.11.4 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/); no adapter trimming was required, and reads had an average Phred score >25. Genome data for strain GFKo1 were assembled using Megahit v1.2.9 (options: --min-contig-len 500 r), with only contigs ≥500 nt in length retained. CheckM2 v0.1.3 [29] was used to determine the completeness and contamination of the genome sequence. Bakta v1.4.2 (database 3.1) [30] was used to annotate predicted genes within the genome.

Identification of genomes

Ribosomal multilocus sequence typing (rMLST [31]) was used to identify the closest relative of strain GFKo1. OAT:OrthoANI v0.5.0 [32] was used to determine orthologous ANI (oANI) values for the genome with publicly available *L. amnigena* genomes and type strains of closest relatives. The identities of publicly available genome sequences of *L. amnigena* [downloaded from National Center for Biotechnology Information (NCBI) GenBank on 19 March 2023; Table 1] were confirmed by comparison (oANI) with the genome sequences of the type strains of the genus. These genomes were checked, annotated and identified as described above. Sourmash v4.6.1 was used to generate 31-kmer signatures for genomes, which were compared to determine how similar genomes were to one another, and to identify genomes belonging to *L. amnigena sensu stricto* [33]. PhyloPhlAn3 (--diversity medium) was used to confirm the affiliation of all genomes with the genus *Lelliottia*.

Identification of plasmid sequences within the genome of GFKo1

PlasmidFinder [34] was used to search the genome assembly for potential plasmid sequences. The online version of COPLA [35] was used to determine the taxonomy of predicted plasmid sequences.

Identification of AMR genes predicted to be encoded in genomes

Initially, Resistance Gene Identifier (RGI 6.0.1, CARD 3.2.6 [36]) was used to derive information on AMR genes predicted to be encoded in the genome of strain GFKo1. The genome sequence of GFKo1 was also searched for the allele of the chromosomal class C β-lactamase $bla_{\text{LAQ-1}}$ (nucleotide accession MZ497396 [25]) using Geneious Prime v2023.0.1. Based on the result of the $bla_{\text{LAQ-1}}$ search, AMRFinderPlus v3.11.4 (database version 2023-02-23.1) [37] and Bakta annotations were subsequently used for surveying AMR genes in genomes.

A BLASTP database was created using the amino acid sequence of MZ497396. Bakta-annotated protein sequences for all genomes (Table 1) were searched against this sequence, with hits >70% coverage and >70% identity retained. The 'hit' protein sequences were extracted from the .faa Bakta-annotated files using Biostrings v2.64.0 (R v4.3.1, RStudio v2023.06.1) and used to create a multiple-sequence alignment (Clustal Omega v1.2.2; Geneious Prime v2023.0.1) with the protein sequences of the 12 AmpC β-lactamases (ACT-12, ACT-22, BIL-1, CMY-2, CMY-20, LAT-1, CFE-1, YRC-1, MIR-1, MIR-23, ACT-6, ACT-10) included in the study in which

the functionality of the bla_{LAQ-1} protein was demonstrated [25]. A phylogenetic tree was created from the sequence alignment using PhyML v3.3.20180621 (Blosum62 matrix) [38], with bootstrap values determined based on 100 replications. The tree was visualized using iToL v6 [39] with additional annotations made using Adobe Illustrator.

Identification of terminator sequences

Potential transcriptional terminator sequences were identified using the online tool iTErm-PseKNC [40].

Table 1. Sequence summary statistics for Bakta-annotated genomes included in this study

Strain	Accession	Source	Size (bp)	Contigs	GC content (%)	N50	CDS	CheckM2	
								Completeness (%)	Contamination (%)
M1325/93/1 (=GFKo1)	JAUBKL000000000	Porpoise lung, UK	4294992	200	53.1	46243	3954	100	0.06
155047^T	GCA_022171985	Human sputum, PR China	4990088	98	53.7	358667	4707	100	0.20
NCTC 12124^T	GCA_900635465	Soil	4471442	1	52.9	4471442	4572	100	0.23
6331-17^T	GCA_002923025	Water, Germany	4774414	37	54.2	202682	4474	100	0.00
CCUG 25894^T	GCA_004115925	Elm tree, USA	4616251	67	54.8	236780	4293	100	0.05
PFL01^T	GCA_002271215	Jogaejeotgal, Republic of Korea	4603334	1	54.2	4603334	4237	100	0.01
LST-1	CP063663	*Stevia*, PR China	3576481	1	41.1	3576481	3187	100	0.03
JCM 17292^T	GCA_001550155	Sediment, Arabian Sea	4459111	26	40.9	658688	4004	100	0.19
2017H1G6	GCA_004331765	Soil, Denmark	4606148	90	52.7	134684	4343	100	0.01
4928STDY7071390	GCA_902160115	Human faeces, UK	4467891	28	55.3	476430	4119	100	0.00
A167	GCA_021498285	Soil, Netherlands	4662149	2	52.8	4520659	4344	100	0.05
ENT01	GCA_025641975	Soil, USA	4716124	59	52.9	212085	4402	100	1.32
ERR1430553*	GCA_938039995	Human faeces, PR China	4361353	909	53.0	5972	4272	90.45	4.58
ERR1430553*	GCA_905202905	Human faeces, PR China	3854042	799	53.4	5991	3704	88.98	5.15
ERR5094855*	GCA_947072025	Rainbow trout gut, France	4359307	65	52.9	139247	4050	99.37	0.65
FDAARGOS 1444	GCA_019047465	Unknown	4505532	1	52.8	4505532	4169	100	0.15
FDAARGOS 1446	GCA_019048185	Unknown	4914411	5	52.6	4591698	4772	100	1.27
FDAARGOS_1445	GCA_019355955	Unknown	4599109	2	52.8	4504790	4287	100	0.06
FDAARGOS_395	GCA_002393405	Soil, USA	4469608	1	52.9	4469608	4130	100	0.01
INSAq176	GCA_021441185	Fish, Portugal	4422149	193	53.2	58074	4147	95.84	0.07
JUb66	GCA_003752235	Unknown	4572787	1	52.9	4572787	4205	100	0.02
P13	GCA_023970615	Pig (sewage), PR China	4622385	2	52.9	4555627	4316	100	0.90
PTJIIT1005	GCA_022352085	Water, India	4550713	71	52.9	298940	4250	100	0.08
TZW12	GCA_016771075	Water, Germany	4694183	26	52.5	415957	4420	100	0.00
TZW13	GCA_016770995	Water, Germany	4830285	26	52.5	337333	4622	100	0.05
TZW14	GCA_016770935	Water, Germany	4516381	17	52.8	731232	4206	100	0.01
TZW15	GCA_016770975	Water, Germany	4756711	36	52.6	346396	4485	100	0.03
TZW16	GCA_016770955	Water, Germany	4756331	35	52.6	346396	4481	100	0.03
UMA3121	GCA_013337605	Forest soil, Portugal	4420612	19	52.9	559149	4091	100	0.00
ZB04	GCA_001652505	Midgut of silkworm, PR China	4616122	1	54.3	4616122	4205	100	0.03

*MAGs; full names ERR1430553_bin.131_CONCOCT_v1.1_MAG, ERR1430553-bin.48 and ERR5094855_bin.4_metaWRAP_v1.3_MAG.

RESULTS

Characteristics of genome of GFKo1

Strain GFKo1 was recovered from the lung of a harbour porpoise that stranded in 1993. Although originally thought to represent a strain of *K. oxytoca*, *rpoB* gene sequence analysis performed in the laboratory at Nottingham Trent University showed that the strain was a representative of *L. amnigena* [28]. This identification was supported by API 20E data (read after 24 and 48 h; code 1305173: *Enterobacter amnigenus* 1 90.4%) and by MALDI-TOF MS with scores that reached 2.48, well above the 2.0 cut-off for species identification.

As *L. amnigena* has not previously been associated with marine mammals and there are few genome sequences available for the species, we generated the draft genome sequence of strain GFKo1 (20× coverage). The genome comprised 4 294 992 bp across 200 contigs (N50 46 243), and was predicted to encode 3954 coding sequences, 80 tRNA, 1 tmRNA and 6 rRNA genes (Table 1). This information, together with its high completeness and low contamination (Table 1), demonstrated that GFKo1's genome was of high quality [41]. PlasmidFinder predicted contigs 181 and 182 (GenBank numbering, PGAP output file GFKo100000000) to encode plasmid sequences, both identified as Col440I-like (fragments within both sequences were related to an unnamed plasmid identified in *Klebsiella pneumoniae* FDAARGOS_440, GenBank accession CP023920.1). COPLA identified the plasmid sequences as belonging to PTU-E3. Among the nine genes contig 181 was predicted to encode were MobC, MbeB and MbeD plasmid mobilization proteins. Contig 182 was predicted to encode only two proteins: a Rop family plasmid primer RNA-binding protein and a hypothetical protein. Given their identities based on PlasmidFinder and COPLA, it is likely that contigs 181 and 182 are part of the same mobilizable plasmid, but a complete sequence would be required to confirm this.

rMLST [31, 42] identified GFKo1 as *L. amnigena* (100% identity). This is a rapid method that indexes variation of the 53 genes encoding bacterial ribosome protein subunits to integrate microbial taxonomy and typing. oANI analysis of GFKo1's genome against the genomes of type strains of the genus *Lelliottia* confirmed GFKo1 as a strain of *L. amnigena*, sharing 98.21% oANI with the type strain (NCTC 12124^{T}, assembly accession GCA_900635465) of the species [43] (Fig. 1a).

Curation of *Lelliottia* genome dataset

We downloaded the GenBank genome assemblies of all *Lelliottia* type strains (*n*=5) and all *L. amnigena* (*n*=22, excluding *L. amnigena* type) strains from NCBI GenBank (Table 2). All were checked for completeness and contamination using CheckM2 (Table 1). Except for metagenome-assembled genome (MAG) ERR1430553, all were of high quality (<5% contamination, >90% complete) [41].

rMLST was used to provide tentative identifications for the *Lelliottia* genome sequences. As can be seen in Table 2, of the 23 genomes identified by NCBI as *L. amnigena*, only 19 were identified as *L. amnigena* with 100% support by PubMLST, with 2 of the MAGs (ERR1430553, ERR1430553) identified as *L. amnigena* with low support scores. Strain 4928STDY7071390 (accession GCA_902160115) was identified as *L. nimipressuralis* (93% support), while strain ZB04 was identified as *Huaxiibacter chinensis* (96% support). Notable was identification of the proposed type strain of '*L. steviae*' [8] as *Pseudoalteromonas arabiensis* (100% support). *L. jeotgali* is an earlier heterotypic synonym of *L. aquatilis* [9], so we would expect the genomes of these species to share high support scores.

oANI analysis was undertaken to confirm identities of genomes (Fig. S1, available with the online version of this article). Identities determined by rMLST were confirmed for all genomes, except for strain A167 (accession GCA_021498285). An ANI of <95% (93.61%) with the genome of the type strain of *L. amnigena* suggests that this strain represents a novel species of *Lelliottia* [43]. The genome of *L. jeotgali* shared 98.78% oANI with that of *L. aquatilis*. Sourmash is a rapid method for computing hash sketches from genomic DNA sequences, and comparing them to each other. A comparison for sourmash signatures generated for all strains supported our findings from rMLST and oANI analyses (Fig. 1b). The sourmash analysis also confirmed the affiliation of GFKo1 with *L. amnigena*.

The genomes (*n*=19) of *L. amnigena* identified by rMLST to be *L. amnigena* (100% support) and sharing oANI of >95% with the genome of the type strain of *L. amnigena* were included in a phylogenetic analysis with the genomes of the type strains of *L. aquatilis* and *L. nimipressuralis* (Fig. 1c). All isolate-derived genomes clustered with the type strain of *L. amnigena*, while the MAG-derived sequence ERR5094855 clustered with *L. aquatilis* and *L. nimipressuralis*. The phylogenetic analysis confirmed the affiliation of GFKo1 with *L. amnigena*.

Carriage of *bla*$_{LAQ-1}$-like genes by *L. amnigena*

RGI/CARD analysis (loose, strict and perfect matches with protein sequences) showed that strain GFKo1's genome encoded no AMR genes. A pairwise alignment of GFKo1's genome with the reference allele sequence of *bla*$_{LAQ-1}$ [25] showed that GFKo1 encoded this class C β-lactamase (Fig. S2), sharing 99.3% nucleotide and 99.5% amino acid pairwise identity with the reference sequence (accession MZ497396). In agreement with [25], we found that *bla*$_{LAQ-1}$ encoded by GFKo1 had the obligatory serine active site of the β-lactamase catalytic motif S–V–S–K (serine–valine–serine–lysine) at positions 83–86, the typical class C β-lactamase motif Y–A–N (tryptophan–alanine–asparagine) at positions 169–171, D/E (a peptide segment containing two dicarboxylic

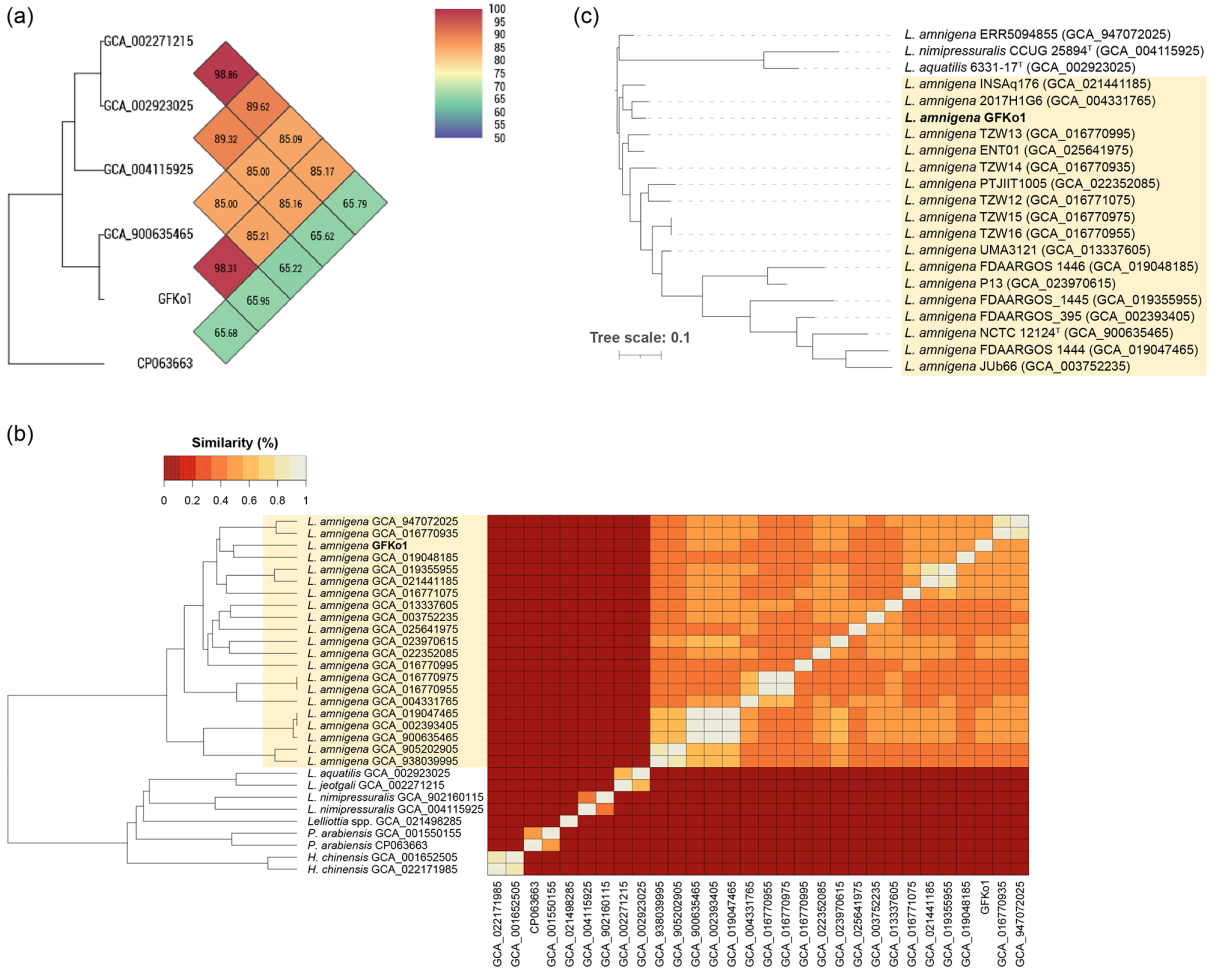

Fig. 1. Strain GFKo1 is a representative of *L. amnigena*. (a) Heatmap generated by OAT:OrthoANI showing the oANI between GFKo1 and strains listed as type strains of *Lelliottia* species with valid and non-valid names. GFKo1 shares highest oANI (%) with the type strain of *L. amnigena* (accession assembly GCA_900635465). (b) Heatmap with unidirectional clustering showing the similarity of sourmash signatures across all genomes included in this study. The lighter the colour of the block on the heatmap, the more similar the two corresponding genome signatures. (c) RAXmL (best tree) generated by PhyloPhlAn3 from the proteomes of high-quality (>90% completeness, <5% contamination; Table 1) genome sequence data for the genus *Lelliottia*. The tree was rooted on the clade containing *L. nimipressuralis* and *L. aquatilis*. Scale bar, average number of amino acid substitutions per position. (b, c) The clade highlighted in light yellow represents *L. amnigena sensu stricto*.

amino acids) at positions 236–238 and the conserved triad K–T–G (lysine–threonine–glycine) at positions 334–336 (Fig. S3). Comparison of the genomic region surrounding $bla_{\text{LAQ-1}}$ revealed a 275 bp intergenic deletion between the *envC* and *empA* genes encoded by strain GFKo1. Analysis of this region revealed the presence of three predicted bi-directional transcriptional terminators that are missing from the genome of GFKo1 (Fig. S4). These are characterized by containing both a poly(A) and poly(T) tract, enabling the terminator to function in both directions.

It is important to note that Bakta had annotated the bla_{LAQ} gene on contig 81 of GFKo1's genome (locus tag GFKo1_06635). Among its databases, Bakta uses the NCBI Antimicrobial Resistance Gene Finder (AMRFinderPlus) [37] to annotate AMR-associated genes in microbial genomes. In addition to a $bla_{\text{LAQ-1}}$-like gene, AMRFinderPlus predicted GFKo1 to encode *vat* (Vat family streptogramin A *O*-acetyltransferase; GFKo1_06890), *catA* (type A chloramphenicol *O*-acetyltransferase; GFKo1_12820) and *oqxB* (multidrug efflux RND transporter permease subunit OqxB; GFKo1_19950). Bakta also predicted GFKo1 to encode the following AMR-associated genes: multidrug efflux MATE transporter EmmdR (GFKo1_03505); multidrug efflux MFS transporter EmrD (GFKo1_03800); Bcr/CflA family efflux transporter (GFKo1_04835); MdtK family multidrug efflux MATE transporter (GFKo1_04850); MATE efflux family protein (GFKo1_06250); multidrug efflux pump accessory protein AcrZ (GFKo1_15865); macrolide-specific efflux protein MacA (GFKo1_16470); putative aminoglycoside efflux pump (GFKo1_16810); multidrug efflux pump subunit AcrB (GFKo1_17175); multidrug efflux RND transporter periplasmic adaptor subunit AcrA (GFKo1_17180); multidrug efflux transporter transcriptional repressor AcrR (GFKo1_17185).

Table 2. Species identities of genomes included in this study as determined using different methods

Strain	Accession	NCBI ID	rMLST ID, % support	oANI with type strain genome
M1325/93/1 (GFKo1)	JAUBKL000000000	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.31%
155047^T	GCA_022171985	*Huaxiibacter chinensis*	*H. chinensis* 100%	*H. chinensis* 100%
NCTC 12124^T	GCA_900635465	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 100%
6331-17^T	GCA_002923025	*L. aquatilis*	*L. aquatilis* 100%	*L. aquatilis* 100%
CCUG 25894^T	GCA_004115925	*L. nimipressuralis*	*L. nimipressuralis* 100%	*L. nimipressuralis* 100%
PFL01^T	GCA_002271215	*L. jeotgali*	*L. aquatilis* 90%	*L. jeotgali* 100%
LST-1	CP063663	'*L. steviae*'	*P. arabiensis* 100%	*P. arabiensis* 99.13%
JCM 17292^T	GCA_001550155	*P. arabiensis*	*P. arabiensis* 100%	*P. arabiensis* 100%
2017H1G6	GCA_004331765	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.41%
4928STDY7071390	GCA_902160115	*L. amnigena*	*L. nimipressuralis* 93%	*L. nimipressuralis* 98.15%
A167	GCA_021498285	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 93.65%
ENT01	GCA_025641975	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.29%
ERR1430553*	GCA_938039995	*L. amnigena*	*L. amnigena* 54%	*L. amnigena* 99.15%
ERR1430553*	GCA_905202905	*L. amnigena*	*L. amnigena* 57%	*L. amnigena* 99.20%
ERR5094855*	GCA_947072025	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.32%
FDAARGOS 1444	GCA_019047465	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 99.97%
FDAARGOS 1446	GCA_019048185	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.32%
FDAARGOS_1445	GCA_019355955	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.45%
FDAARGOS_395	GCA_002393405	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 99.97%
INSAq176	GCA_021441185	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.42%
JUb66	GCA_003752235	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.40%
P13	GCA_023970615	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.87%
PTJIIT1005	GCA_022352085	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.85%
TZW12	GCA_016771075	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.45%
TZW13	GCA_016770995	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.30%
TZW14	GCA_016770935	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.24%
TZW15	GCA_016770975	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.42%
TZW16	GCA_016770955	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.42%
UMA3121	GCA_013337605	*L. amnigena*	*L. amnigena* 100%	*L. amnigena* 98.44%
ZB04	GCA_001652505	*L. amnigena*	*H. chinensis* 96%	*H. chinensis* 99.76%

*MAGs; full names ERR1430553_bin.131_CONCOCT_v1.1_MAG, ERR1430553-bin.48 and ERR5094855_bin.4_metaWRAP_v1.3_MAG.

A BLASTP search of the predicted proteins in each of the genomes listed in Table 1 against the amino acid sequence (380 aa) of the Bla_{LAQ-1} reference sequence identified one hit in each genome that shared >70% identity and 100% coverage with MZ497396 (Table S1, available with the online version of this article). The 'hit' sequences were extracted from the Bakta annotation files (available from figshare as Supplementary Material) for the genomes and used to create a multiple sequence alignment with the AmpC reference sequences included in the original characterization of bla_{LAQ-1} [25]. A phylogenetic analysis (maximum likelihood) demonstrated that all of the *L. amnigena* sequences clustered together (Fig. 2), sharing pairwise identity values of 98.16–99.47% with Bla_{LAQ-1} of P13 and 97.63–100% with each other (Table S2), and high bootstrap support (97%). The sequence of strain A167 (accession GCA_021498285) formed a branch on its own (100% bootstrap support), providing additional support that this strain represents a novel species of *Lelliottia* (93.42% amino acid identity with P13's Bla_{LAQ-1} sequence). The sequences derived from *H. chinensis* strains clustered together but apart from the *L. amnigena* sequences, as did those of *L. nimipressuralis*, and those of *L. aquatilis* and *L. jeotgali* (all with 100% bootstrap support).

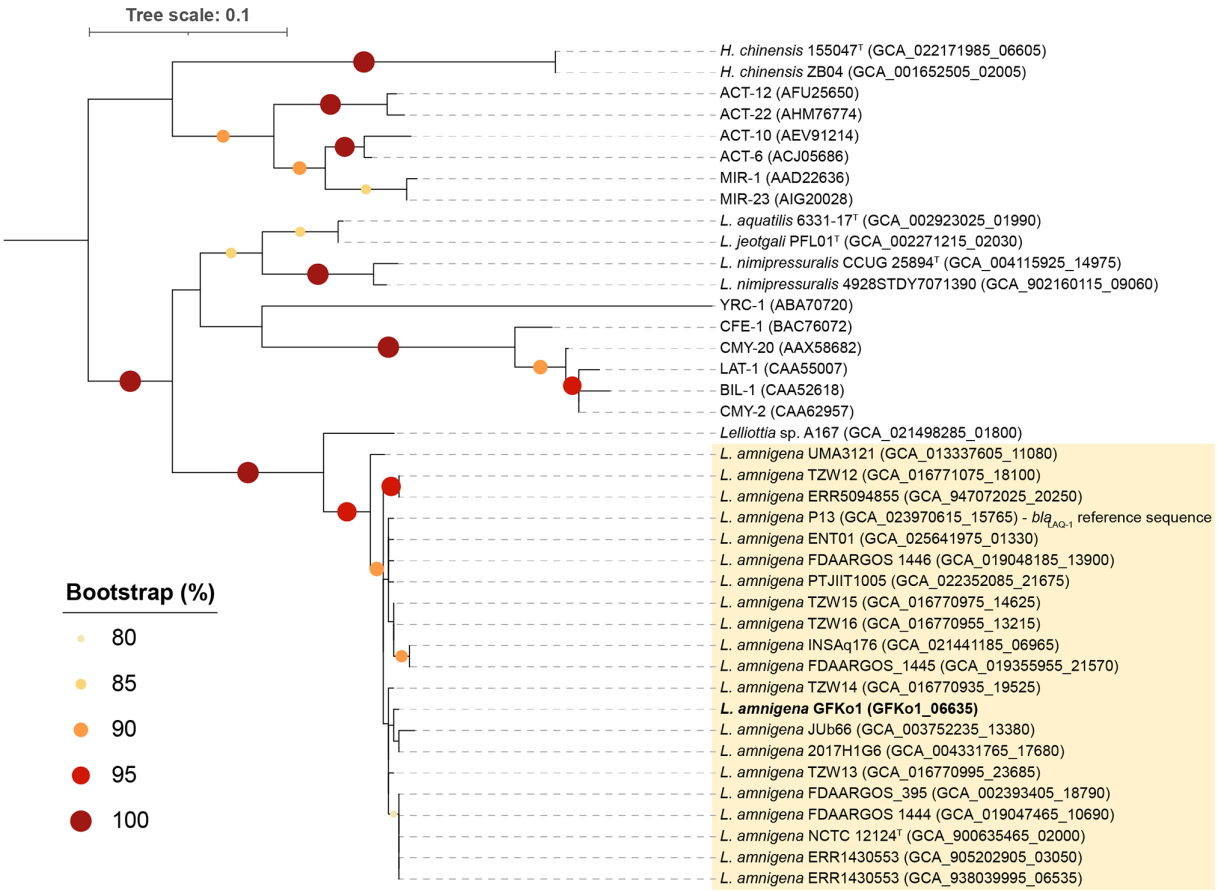

Fig. 2. bla_{LAQ} is a core gene of *L. amnigena*. The Bla_{LAQ-1} sequence of *L. amnigena* P13 represents the reference for this chromosomally encoded AmpC β-lactamase [25]. Twelve other AmpC β-lactamases (ACT-12, ACT-22, BIL-1, CMY-2, CMY-20, LAT-1, CFE-1, YRC-1, MIR-1, MIR-23, ACT-6, ACT-10 [25]) were included in the analysis for comparative purposes; the accessions for the amino acid sequences of these proteins are given in parentheses. The tree was rooted at the midpoint. Scale bar, average number of amino acid substitutions per position. The clade in yellow highlights *L. amnigena sensu stricto* sequences. Bootstrap values >80% (based on 100 replications) are shown on the tree. The multiple sequence alignment used to create this phylogenetic tree is available from figshare as Supplementary Material.

Phenotypic resistance profile of *L. amnigena* GFKo1

Disc diffusion assays were performed against antibiotics from a range of classes to determine the phenotypic resistance profile of *L. amnigena* GFKo1. Strain GFKo1 was found to be clinically sensitive to all antibiotics tested: penicillins (ampicillin, ampicillin–sulbactam, piperacillin, amoxicillin–clavulanate, piperacillin–tazobactam); cephalosporins (cefoxitin, ceftazidime, cefepime, cefotaxime, ceftriaxone); carbapenems (imipenem, meropenem, ertapenem); the monobactam aztreonam; the aminoglycosides amikacin and gentamicin; the fluoroquinolones ciprofloxacin and norfloxacin; the tetracyclines tigecycline and tetracycline; and trimethoprim and sulphamethoxazole–trimethoprim. A full table of results, including zone diameters measured and breakpoints, can be found in Table S3.

DISCUSSION

In this study, we have characterized the genome and AMR genotype/phenotype of a strain of *L. amnigena* (GFKo1) isolated from the lung of a harbour porpoise stranded in 1993. We compared the genome of GFKo1 with genomes of closely related species (Fig. 1, Tables 1 and 2), and demonstrated that bla_{LAQ}, a chromosomally encoded AmpC β-lactamase conferring resistance to penicillin G, ampicillin and several cephalosporins [25], is a core gene of *L. amnigena* (Fig. 2). Phenotypically, GFKo1 was sensitive to all antibiotics it was tested against, including ampicillin, cefotaxime and ceftazidime (Table S3).

Our detailed genome-based identification of *L. amnigena* genomes (n=20 isolates; n=3 MAGs) downloaded from GenBank highlighted misclassification problems with four of the genomes, including that of a proposed type strain for '*L. steviae*' [8] (Fig. 1, Table 2). While the NCBI classifies some genome assemblies as anomalous and excludes them from the RefSeq database

based on a range of different criteria, these assemblies are still available for download from GenBank. *Lelliottia* spp. data within NCBI GenBank are derived from isolates and MAGs, with no information provided as to, for example, the completeness and contamination of the genomes compared with accepted standards [41]. We have previously encountered problems with taxonomic assignments provided by the NCBI (although we acknowledge that annotations are improving and being updated constantly [44]). However, we still recommend that, for informative and accurate comparative genomic analyses to be undertaken, it is important that the genomes of all bacteria retrieved from public repositories are carefully checked for quality and identity before undertaking in-depth analyses.

In addition to identifying bla_{LAQ} as a core gene of *L. amnigena*, we demonstrated that proteins sharing high identity with a range of other AmpC β-lactamases were identified across all genomes included in this study (Fig. 2). Whether these AmpC β-lactamases detected in non-*L. amnigena* genomes are functional remains to be determined. With respect to the Bla_{LAQ} protein of GFKo1, it possessed the canonical motifs and active sites associated with β-lactamase enzymes. Additionally, it shared 99.5% amino acid pairwise identity with LAQ-1 from *L. amnigena* P13 (accession MZ497396). It has been suggested that LAQ-1 from *L. amnigena* P13 confers resistance to a range of β-lactams, including first- to fourth-generation cephalosporins. A recombinant *E. coli* clone of the β-lactamase from a plasmid-borne copy of bla_{LAQ-1} exhibited increased minimum inhibitory concentrations (MICs) to a range of antibiotics, including ampicillin, cefoxitin, cefazolin, ceftazidime, cefepime, aztreonam, ticaracillin, piperacllin and cloxacillin. However, these increased MICs only resulted in clinical resistance to ampicillin, cefoxitin and cefazolin according to EUCAST guidelines. Despite the high level of sequence similarity between the bla_{LAQ} gene of GFKo1 and that from P13, *L. amnigena* GFKo1 was sensitive to all antibiotics tested in our study. Genomic alignment of the two strains showed a high level of sequence similarity in the region immediately upstream of the bla_{LAQ-1} gene, suggesting that lack of activity is not due to a mutation (or mutations) in the promoter region. However, further analysis of the genomic region surrounding bla_{LAQ-1} revealed a 275 bp intergenic deletion between the *envC* and *empA* genes upstream of bla_{LAQ-1} in strain GFKo1. Analysis of this region revealed the presence of three predicted bi-directional transcriptional terminators that are missing from the genome of GFKo1. As these terminators appear to be bi-directional, characterized by the presence of both a poly(A) and poly(T) tract, it is likely that their absence in GFKo1 will affect transcription both upstream and downstream of these sites.

Despite bla_{LAQ} being a core gene of all sequenced *L. amnigena* isolates, it is evident that broad-spectrum resistance to β-lactam antibiotics is not a uniform feature of the species. Resistance to penicillins is reported frequently, but resistance to specific cephalosporins is highly variable [25, 45–47]. Genome sequence data are rarely available for the strains characterized in these studies, making it difficult to determine the genotypic factors that contribute to the observed resistant phenotypes.

In summary, we show that the chromosomally encoded AmpC β-lactamase bla_{LAQ} is a core gene of *L. amnigena*. However, presence of the bla_{LAQ} gene does not always correlate with phenotypic resistance to β-lactam antibiotics. Resistance to specific cephalosporins appears to be highly variable across the species. The mechanisms controlling bla_{LAQ} expression, and the degree to which bla_{LAQ} contributes to phenotypic resistance, require further investigation. Studies involving the cloning and expression of diverse bla_{LAQ} genes in genetic backgrounds free from other resistance markers will help elucidate the specificity of these novel β-lactamases and their role in *L. amnigena*.

Funding information
This work used computing resources provided through the Research Contingency Fund of Nottingham Trent University.

Author contributions
D.N., G.F., L.H. – conceptualization, data curation, investigation, methodology, validation, writing (original draft; review and editing). D.N., L.H. – formal analysis, visualization. L.H. – project administration, software, resources, funding acquisition.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000694.v2.3
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Jana Katharina Schniete; Leibniz Universitat Hannover Naturwissenschaftliche Fakultat, Institut fuer Mikrobiologie, Herrenhaeuser Strasse 2, Geb 4104, GERMANY, Hannover

Date report received: 20 October 2023
Recommendation: Accept

Comments: Thank you for addressing the comments from the reviewers. In your point by point response, in your answer to reviewer 1 I noticed that you refer in the last sentence to a number of references, do you meant to say in silico analyses? At least reference 10 and 22 which I checked in terms of the bla gene I did not find phenotypic analysis. I quote the paragraph here: "We are unable to find additional publications which report on concordance/discordance between genotypic predictions and phenotypic drug resistances in this species. We highlight this as an issue in the discussion. References 10, 21, 22 and 38-40 all describe phenotypic resistances in Lelliottia spp." You may want to address this prior to publication to the platform.

SciScore report

https://doi.org/10.1099/acmi.0.000694.v2.1
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iThenticate report

https://doi.org/10.1099/acmi.0.000694.v2.2
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Author response to reviewers to Version 1

REVIEWERS' COMMENTS

Reviewer 1

In this brief report the authors describe the genome analyses of a *Lelliottia amnigena*isolate obtained from a dolphin with the main focus on the antibiotic resistance profile, including phenotypic resistance tests. The authors compare the genome with publically available genomic data from other *Lelliottia*strains. In general, the manuscript is well-written and the results are adequately presented. The two tables present the necessary information of the strains included in the study. The figures are informative with all relevant information included. The main key findings of the manuscript, the characterization of the genome, the taxonomic analyses, as well as AMR analyses and the phenotypic resistance profile are well described. The discussion sums up all relevant finding, yet, it somehow misses the discussion of the results in comparison with other studies. Here, the relevant literature (already included in the manuscript) should be also discussed. E.g. references 10 and 22 also showed the presence of the AmpC gene, although the strain(s) showed no phenotypic resistance, comparable to the results of the manuscript. Thus, these references (and possibly others) should be included into the discussion.

Specific comments:

Line 62: Please cite additional relevant literature reports on AMR genes in *Lelliottia*in addition to the two mentioned papers.

Reference 10 (Reitter C, Neuhaus K, Hügler M) reports draft genome sequences of *Enterobacter*spp., *Lelliottia*spp., and *Serratia*spp. and is a short announcement. The analyses of the strains are purely bioinformatics based. Although the authors do state that all

genome sequences were found to encode the *ampC*gene, they did not perform any phenotypic characterisation of the strains and therefore we feel unable to comment on this aspect.

Reference 22 (El Zowalaty ME, et al.) reports the draft genome sequence of a rare *Lelliottia nimipressuralis*strain. The only antimicrobial susceptibility test reported in their manuscript is the MIC of colistin using the broth microdilution method. We also feel we are unable to draw any conclusions relating to AmpC production and beta-lactam resistance as it appears the authors did not perform these tests.

We are unable to find additional publications which report on concordance/discordance between genotypic predictions and phenotypic drug resistances in this species. We highlight this as an issue in the discussion. References 10, 21, 22 and 38-40 all describe phenotypic resistances in *Lelliottia*spp.

Line 105: "Accurate identification of genomes". I feel, that "accurate" is not the right terminology here and suggest removing the word.

We have made the requested amendment.

Reviewer 2

1. Methodological rigour, reproducibility and availability of underlying data

The authors have satisfactorily described their isolation procedures and their methodology for phenotypic characterisation such that it could be reproduced - the methodologies themselves are sound and rational. Likewise for their descriptions of library preparation and sequencing and their assembly and annotation processes. Could the authors please change "accurate identification of genomes" to more clearly illustrate that this section refers to taxonomic identification. In their AMR gene identification, could the authors please state the version of R and RStudio they carried out their BioStrings analysis work in. The paper is methodologically sound.

We have made the requested amendments.

2. Presentation of results

The authors have satisfactorily described the genome of GFKo1, however I would like to see a map of the chromosome. Is there any indication of the presence of plasmids from the assembly? When referring to ANI in this section and throughout the text, could the authors be explicit at all times that they mean OrthoANI? The reason I ask is that oANI is its own metric which, whilst suitable for this study, would likely produce slightly different results if different algorithms were used. In line 174, could the authors provide the ANI as a supplementary figure?

As the genome assembly of GFKo1 is in multiple contigs, and the plasmid carriage of *Lelliottia*spp. is poorly defined, we do not think it appropriate to include a map of the chromosome in the manuscript. If we had a complete assembly for GFKo1's genome we would have included a Genovi map or similar.

We have included plasmid data, with relevant information in Methods and Results.

We have now made it clear throughout the text that oANI was used. The results from the full oANI analysis have been included as a Supplementary Figure.

In figure one, could the authors please reorder or reletter the panels such that they read A, B, C from left to right instead of A, C, B in the figure's current format. In panel 1b, could the authors please redraw the heatmap with black borders between cells, for better readability. In figure 1c, could the authors indicate bootstrap values on their phylogenetic tree? In figure 2, bootstrap values of the blalaq locus are indicated by circle size - this is difficult to interpret as the distinction between e.g. 80 and 85% is not immediately clear. I suggest that the authors use a colour scale to represent these values instead. Presentation of the antimicrobial resistance data is adequate, although I feel this would have been complemented with data from resistant Leliotta as a comparison.

When discussing the alignment of the region surrounding blalaq¬ in figure S2, could the authors comment on the ~300 bp intergenic deletion between envC and empA - has the isolate lost any terminators etc from this region?

The panels are arranged in Figure 1 to make best use of space. We do not think it necessary to reorder the panels. The heatmap of Figure 1b has been redrawn as requested.

Bootstrap values in Figure 2 have been represented by a colour scale as requested.

We have analysed the intergenic region mentioned and found three potential terminators. This information has been included in the results (new supplementary figure) and potential impact added in the discussion.

3. How the style and organization of the paper communicates and represents key findings

The paper is logically organized and presents a sensible story, which adequately and clearly communicates its findings - I have no issue with the manuscript's organisation

Thank you for your positive feedback.

4. Literature analysis or discussion

The introduction of the paper adequately summarises the literature surrounding Lelliottia genomics and taxonomy. They sufficiently describe the genus' ecology, what is known about AMR phenotypes of its members, and this works context within the authors' wider projects and research interests. The authors adequately discuss how the results presented fit into current understanding of literature, and correctly acknowledge the importance confirming the taxonomy of publicly available genomes. I particularly enjoyed the section describing the variable AMR phenotype of the species.

Thank you for your positive feedback.

5. Any other relevant comments

The manuscript is adequate for publication, pending the quick fixes to the clarity of their methodology and figures. I feel like the authors have missed a trick by not performing a qPCR of blalaq, to test if it is expressed under laboratory conditions, however that experiment is not key to this paper.

Thank you for your positive feedback. We plan to get a future masters student to look at expression of the gene under laboratory conditions, as it is of interest to us but not the focus of the reported work.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000694.v1.5
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Date report received: 10 October 2023
Recommendation: Minor Amendment

Comments: This study would be a valuable contribution to the existing literature. The reviewers have highlighted minor concerns with the work presented. Please ensure that you address their comments.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000694.v1.4
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Date report received: 07 October 2023
Recommendation: Minor Amendment

Comments: 1. Methodological rigour, reproducibility and availability of underlying data The authors have satisfactorily described their isolation procedures and their methodology for phenotypic characterisation such that it could be reproduced - the methodologies themselves are sound and rational. Likewise for their descriptions of library preparation and sequencing and their assembly and annotation processes. Could the authors please change "accurate identification of genomes" to more clearly illustrate that this section refers to taxonomic identification. In their AMR gene identification, could the authors please state the version of R and RStudio they carried out their BioStrings analysis work in. The paper is methodologically sound. 2. Presentation of results The authors have satisfactorily described the genome of GFKo1, however I would like to see a map of the chromosome. Is

there any indication of the presence of plasmids from the assembly? When referring to ANI in this section and throughout the text, could the authors be explicit at all times that they mean OrthoANI? The reason I ask is that oANI is its own metric which, whilst suitable for this study, would likely produce slightly different results if different algorithms were used. In line 174, could the authors provide the ANI as a supplementary figure? In figure one, could the authors please reorder or reletter the panels such that they read A, B, C from left to right instead of A, C, B in the figure's current format. In panel 1b, could the authors please redraw the heatmap with black borders between cells, for better readability. In figure 1c, could the authors indicate bootstrap values on their phylogenetic tree? In figure 2, bootstrap values of the blalaq locus are indicated by circle size - this is difficult to interpret as the distinction between e.g. 80 and 85% is not immediately clear. I suggest that the authors use a colour scale to represent these values instead. Presentation of the antimicrobial resistance data is adequate, although I feel this would have been complemented with data from resistant Leliotta as a comparison. When discussing the alignment of the region surrounding blalaq¬ in figure S2, could the authors comment on the ~300 bp intergenic deletion between envC and empA - has the isolate lost any terminators etc from this region? 3. How the style and organization of the paper communicates and represents key findings The paper is logically organized and presents a sensible story, which adequately and clearly communicates its findings - I have no issue with the manuscript's organisation 4. Literature analysis or discussion The introduction of the paper adequately summarises the literature surrounding Lelliottia genomics and taxonomy. They sufficiently describe the genus' ecology, what is known about AMR phenotypes of its members, and this works context within the authors' wider projects and research interests. The authors adequately discuss how the results presented fit into current understanding of literature, and correctly acknowledge the importance confirming the taxonomy of publicly available genomes. I particularly enjoyed the section describing the variable AMR phenotype of the species. 5. Any other relevant comments The manuscript is adequate for publication, pending the quick fixes to the clarity of their methodology and figures. I feel like the authors have missed a trick by not performing a qPCR of blalaq¬, to test if it is expressed under laboratory conditions, however that experiment is not key to this paper.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
No: No animal work

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000694.v1.3
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Anonymous.

Date report received: 04 October 2023
Recommendation: Minor Amendment

Comments: In this brief report the authors describe the genome analyses of a Lelliottia amnigena isolate obtained from a dolphin with the main focus on the antibiotic resistance profile, including phenotypic resistance tests. The authors compare the genome with publically available genomic data from other Lelliottia strains. In general, the manuscript is well-written and the results are adequately presented. The two tables present the necessary information of the strains included in the study. The figures are informative with all relevant information included. The main key findings of the manuscript, the characterization of the genome, the taxonomic analyses, as well as AMR analyses and the phenotypic resistance profile are well described. The discussion sums up all relevant finding, yet, it somehow misses the discussion of the results in comparison with other studies. Here, the relevant literature (already included in the manuscript) should be also discussed. E.g. references 10 and 22 also showed the presence of

the AmpC gene, although the strain(s) showed no phenotypic resistance, comparable to the results of the manuscript. Thus, these references (and possibly others) should be included into the discussion. Specific comments: Line 62: Please cite additional relevant literature reports on AMR genes in Lelliottia in addition to the two mentioned papers. Line 105: "Accurate identification of genomes". I feel, that "accurate" is not the right terminology here and suggest removing the word.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000694.v1.1
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iThenticate report

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