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

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Abbreviations: AMR, antimicrobial resistance; ANI, average nucleotide identity; EUCAST, EUropean Committee on Antimicrobial Susceptibility Testing; MAG, metagenome-assembled genome; rMLST, ribosomal multi-locus sequence typing.

Supplementary material associated with this article is available from figshare: https://figshare.com/projects/Lelliottia_amnigena_characterization/174210.

The whole-genome sequence data generated for this study are available from BioProject PRJNA979992.

ABSTRACT

Strain M1325/93/1 (= 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 the recently described chromosomally-encoded AmpC β-lactamase bla_{LAQ} is a core gene of *L. amnigena*, and suggest 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 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.*

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*") (Brady *et al.* 2013; Kämpfer *et al.* 2018; Yuk *et al.* 2018; Lin *et al.* 2022). *Lelliottia aquatilis* represents a later heterotypic synonym of *L. jeotgali*, based on average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization analyses (Wu and Zong 2019).

Lelliottia spp. have been associated with the commensal microbiota of flies and the Asian tiger mosquito (Guégan *et al.* 2020; Wiktorczyk-Kapischke *et al.* 2022), and isolated from fresh and waste water, soil, plants, air samples and fish (Heinle *et al.* 2018; Kämpfer *et al.* 2018; Yuk *et al.* 2018; Salgueiro *et al.* 2020; Reitter, Neuhaus and Hügler 2021; Leister and Hügler 2022; Thakur and Gauba 2022; Tran *et al.* 2022; Bilous *et al.* 2023; Suescun-Sepulveda, Rondón González and Fuentes Lorenzo 2023). 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 (Osei *et al.* 2022). Only rarely have *L. amnigena* and *L. nimipressuralis* been associated with opportunistic disease in humans (Leal-Negredo *et al.* 2017; Martín Guerra, Martín Asenjo and Dueñas Gutiérrez 2018; Choi *et al.* 2021; Legese *et al.* 2022). 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-1}, conferring resistance to ampicillin and several cephalosporins was recently described for an *L. amnigena* strain isolated from animal farm sewage in China (Li *et al.* 2022; El Zowalaty *et al.* 2023).

As part of a study of veterinary isolates thought to belong to the *Klebsiella oxytoca* complex (Smith-Zaitlik *et al.* 2022), we identified several atypical strains that were shown by *rpoB* gene sequence analysis to represent a range of different *Enterobacteriaceae* (Smith-Zaitlik 2021). Here, we report on one such strain recovered from the lung of a harbour porpoise (*Phocoena phocoena*). Using genome sequence data and comparative analyses, we demonstrate this is a strain of *L. amnigena* and compare its AMR gene profile with those of publicly available sequence data for the species.

MATERIALS AND 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 made 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-ionisation time-of-flight mass spectroscopy (MALDI-TOF) 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 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 (Chklovski *et al.* 2022) was used to determine the completeness and contamination of the genome sequence. Bakta v1.4.2 (database 3.1) (Schwengers *et al.* 2021) was used to annotate predicted genes within the genome.

Accurate identification of genomes. Ribosomal multi-locus sequence typing (rMLST; (Jolley *et al.* 2012)) was used to identify the closest relative of strain GFKo1. OAT:OrthoANI v0.93.1 (Lee *et al.* 2016) was used to determine ANI values for the genome with publicly available *L. amnigena* genomes and type strains of closest relatives. Identities of publicly available genome sequences of *L. amnigena* (downloaded from NCBI GenBank on 19 March 2023; **Table 1**) were confirmed by comparison (OAT:OrthoANI) 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 with one another to determine how similar genomes were to one another, and to identify genomes belonging to *L. amnigena sensu stricto* (Brown

and Irber 2016). PhyloPhlAn3 (--diversity medium) was used to confirm the affiliation of all genomes with the genus *Lelliottia*.

Identification of AMR genes predicted to be encoded in genomes. Initially, the Resistance Gene Identifier [RGI 6.0.1, CARD 3.2.6; (Alcock *et al.* 2020)] 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_{LAQ-1} (nucleotide accession MZ497396; (Li *et al.* 2022)) using Geneious Prime v2023.0.1. Based on the result of the bla_{LAQ-1} search, AMRFinderPlus v3.11.4 (database version 2023-02-23.1) (Feldgarden *et al.* 2022) 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 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 (Li *et al.* 2022). A phylogenetic tree was created from the sequence alignment using PhyML v3.3.20180621 (Blosum62 matrix) (Guindon *et al.* 2010), with bootstrap values determined based on 100 replications. The tree was visualized using iToL v6 (Letunic and Bork 2019) with additional annotations made using Adobe Illustrator.

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 done in the laboratory at Nottingham Trent University showed the strain was a representative of *L. amnigena* (Smith-Zaitlik 2021). 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 with scores that reached 2.48, significantly 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 3,954 coding sequences, 80 tRNA, 1 tmRNA and 6 ribosomal RNA genes (**Table 1**). This information, together with its high completeness and low contamination (**Table 1**), demonstrated GFKo1's genome was of high quality (Bowers *et al.* 2017).

rMLST (Jolley *et al.* 2012; Jolley, Bray and Maiden 2018) 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. ANI analysis of GFKo1's genome against the genomes of type strains of the genus *Lelliottia* confirmed GFKo1 as a strain of *L. amnigena*, sharing 98.31 % ANI with the type strain (NCTC 12124^{T}, assembly accession GCA_900635465) of the species (Chun *et al.* 2018) (**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) (Bowers *et al.* 2017).

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 two 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*" (Lin *et al.* 2022) as *Pseudoalteromonas arabiensis* (100 % support). *L. jeotgali* is an earlier heterotypic synonym of *L. aquatilis* (Wu and Zong 2019), so we would expect the genomes of these species to share high support scores.

ANI analysis was undertaken to confirm identities of genomes (not shown). Identities determined by rMLST were confirmed for all genomes, except for strain A167 (accession GCA_021498285). An ANI of <95 % with the genome of the type strain of *L. amnigena* suggests this strain represents a novel species of *Lelliottia* (Chun *et al.* 2018). The genome of *L. jeotgali* shared 98.86 % ANI 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 ANI 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 ANI 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*$_{\text{LAQ-1}}$-like genes by *L. amnigena*

RGI/CARD analysis (loose, strict and perfect matches with protein sequences) showed strain GFKo1's genome encoded no AMR genes. A pairwise alignment of GFKo1's genome with the reference allele sequence of *bla*$_{\text{LAQ-1}}$ (Li *et al.* 2022) showed GFKo1 encoded this class C β-lactamase, sharing 99.3 % nucleotide and 99.5 % amino acid pairwise identity with the reference sequence (accession MZ497396). In agreement with Li *et al.* (2022) we found that *bla*$_{\text{LAQ-1}}$ 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 amino acids) at positions 236–238 and the conserved triad K-T-G (lysine-threonine-glycine) at positions 334–336 (**Supplementary Fig. 1**).

It is important to note that Bakta had annotated the *bla*$_{\text{LAQ}}$ gene on contig 81 of GFKo1's genome (locus tag GFKo1_06635). Among its databases, Bakta uses the NCBI Antimicrobial Resistance Gene Finder (AMRFinderPlus) (Feldgarden *et al.* 2022) 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).

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 (**Supplementary Table 1**). The 'hit' sequences were extracted from the Bakta annotation files (available 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*$_{LAQ-1}$ (Li *et al.* 2022). A phylogenetic analysis (maximum likelihood) demonstrated all 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 (**Supplementary Table 2**), 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).

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 **Supplementary Table 3**.

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 (**Figure 1**, **Table 1** and **Table 2**), and demonstrated that *bla*$_{LAQ}$, a chromosomally-encoded AmpC β-lactamase conferring resistance to penicillin G, ampicillin and several cephalosporins (Li *et al.* 2022), is a core gene of *L. amnigena* (**Figure 2**). Phenotypically, GFKo1 was sensitive to all antibiotics it was tested against, including ampicillin, cefotaxime and ceftazidime (**Supplementary Table 3**).

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*" (Lin *et al.* 2022) (**Figure 1**, **Table 2**). While 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 (Bowers *et al.* 2017). We have previously encountered problems with taxonomic assignments provided by NCBI (though acknowledge annotations are improving and being updated constantly; (Chen *et al.* 2020)). 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 (**Figure 2**). Whether these AmpC β-lactamases detected in non-*L. amnigena* genomes are functional remains to be determined. With respect to the *bla*$_{LAQ}$ gene 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 *Escherichia 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(s) in the promoter region.

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, however resistance to specific cephalosporins is highly variable (Bollet *et al.* 1991; Stock and Wiedemann 2002; Murugaiyan *et al.* 2015; Li *et al.* 2022). Genome sequence data are rarely available for the

strains characterised in these studies, making it impossible 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*.

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GF isolated the strain and performed phenotypic characterization and antibiotic sensitivity test work. DN did all library preparation, sequencing and antibiotic sensitivity testing. LH did all bioinformatics work. DN and LH undertook all data analyses and interpretation. All authors contributed to the writing of the manuscript, and approved the submitted version.

REFERENCES

Alcock BP, Raphenya AR, Lau TTY *et al.* CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res* 2020;**48**:D517–25.

Bilous S, Likhanov A, Boroday V *et al.* Antifungal activity and effect of plant-associated bacteria on phenolic synthesis of *Quercus robur* L. *Plants (Basel)* 2023;**12**:1352.

Bollet C, Elkouby A, Pietri P *et al.* Isolation of *Enterobacter amnigenus* from a heart transplant recipient. *Eur J Clin Microbiol Infect Dis* 1991;**10**:1071–3.

Bowers RM, Kyrpides NC, Stepanauskas R *et al.* Minimum information about a single amplified genome (MISAG) and a metagenome-assembled genome (MIMAG) of bacteria and archaea. *Nat Biotechnol* 2017;**35**:725–31.

Brady C, Cleenwerck I, Venter S *et al.* Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as

Kosakonia cowanii comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Syst Appl Microbiol* 2013;**36**:309–19.

Brown CT, Irber L. sourmash: a library for MinHash sketching of DNA. *Journal of Open Source Software* 2016;**1**:27.

Chen Y, Brook TC, Soe CZ *et al.* Preterm infants harbour diverse *Klebsiella* populations, including atypical species that encode and produce an array of antimicrobial resistance- and virulence-associated factors. *Microb Genom* 2020;**6**, DOI: 10.1099/mgen.0.000377.

Chklovski A, Parks DH, Woodcroft BJ *et al.* CheckM2: a rapid, scalable and accurate tool for assessing microbial genome quality using machine learning. 2022:2022.07.11.499243.

Choi H, Hwang M, Chatterjee P *et al.* Rare *Lelliottia nimipressuralis* from a wound infection case report using whole genome sequencing-based bacterial identification. *Diagn Microbiol Infect Dis* 2021;**101**:115538.

Chun J, Oren A, Ventosa A *et al.* Proposed minimal standards for the use of genome data for the taxonomy of prokaryotes. *Int J Syst Evol Microbiol* 2018;**68**:461–6.

El Zowalaty ME, Falgenhauer L, Forsythe S *et al.* Draft genome sequences of rare *Lelliottia nimipressuralis* strain MEZLN61 and two *Enterobacter kobei* strains MEZEK193 and MEZEK194 carrying mobile colistin resistance *mcr-9* gene isolated from wastewater in South Africa. *J Glob Antimicrob Resist* 2023:S2213-7165(23)00050-4.

Feldgarden M, Brover V, Fedorov B *et al.* Curation of the AMRFinderPlus databases: applications, functionality and impact. *Microbial Genomics* 2022;**8**:000832.

Guégan M, Tran Van V, Martin E *et al.* Who is eating fructose within the *Aedes albopictus* gut microbiota? *Environ Microbiol* 2020;**22**:1193–206.

Guindon S, Dufayard J-F, Lefort V *et al.* New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 2010;**59**:307–21.

Heinle CE, Junqueira ACM, Uchida A *et al.* Complete genome sequence of *Lelliottia nimipressuralis* type strain SGAir0187, isolated from tropical air collected in Singapore. *Genome Announc* 2018;**6**:e00231-18.

Jolley KA, Bliss CM, Bennett JS *et al.* Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. *Microbiology (Reading)* 2012;**158**:1005–15.

Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res* 2018;**3**:124.

Kämpfer P, Glaeser SP, Packroff G *et al. Lelliottia aquatilis* sp. nov., isolated from drinking water. *Int J Syst Evol Microbiol* 2018;**68**:2454–61.

Leal-Negredo A, Castelló-Abieta C, Leiva PS *et al.* Urinary tract infection by *Lelliottia amnigena* (*Enterobacter amnigenus*): an uncommon pathogen. *Rev Esp Quimioter* 2017;**30**:483–4.

Lee I, Ouk Kim Y, Park S-C *et al.* OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* 2016;**66**:1100–3.

Legese MH, Asrat D, Swedberg G *et al.* Sepsis: emerging pathogens and antimicrobial resistance in Ethiopian referral hospitals. *Antimicrob Resist Infect Control* 2022;**11**:83.

Leister C, Hügler M. Genome analysis of *Enterobacter asburiae* and *Lelliottia* spp. proliferating in oligotrophic Drinking water reservoirs and lakes. *Appl Environ Microbiol* 2022;**88**:e0047122.

Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* 2019;**47**:W256–9.

Li A, Yan C, Zhang L *et al.* Characterization and identification of a novel chromosomal class C β-lactamase, LAQ-1, and comparative genomic analysis of a multidrug resistance plasmid in *Lelliottia amnigena* P13. *Front Microbiol* 2022;**13**:990736.

Lin J, Huang K, Huang J-Y *et al. Lelliottia steviae* sp. nov. isolated from *Stevia rebaudiana* Bertoni. *Arch Microbiol* 2022;**204**:475.

Martín Guerra JM, Martín Asenjo M, Dueñas Gutiérrez CJ. Pyonephrosis by *Lelliottia amnigena*. *Med Clin (Barc)* 2018;**151**:419–20.

Murugaiyan J, Krueger K, Roesler U *et al.* Assessment of species and antimicrobial resistance among *Enterobacteriaceae* isolated from mallard duck faeces. *Environ Monit Assess* 2015;**187**:127.

Osei R, Yang C, Cui L *et al.* Isolation, identification, and pathogenicity of *Lelliottia amnigena* causing soft rot of potato tuber in China. *Microb Pathog* 2022;**164**:105441.

Reitter C, Neuhaus K, Hügler M. Draft genome sequences of *Enterobacter* spp., *Lelliottia* spp., and *Serratia* spp., coliform bacteria from drinking water reservoirs and lakes. *Microbiol Resour Announc* 2021;**10**:e0062221.

Salgueiro V, Manageiro V, Bandarra NM *et al.* Bacterial diversity and antibiotic susceptibility of *Sparus aurata* from aquaculture. *Microorganisms* 2020;**8**:1343.

Schwengers O, Jelonek L, Dieckmann MA *et al.* Bakta: rapid and standardized annotation of bacterial genomes via alignment-free sequence identification. *Microb Genom* 2021;**7**, DOI: 10.1099/mgen.0.000685.

Smith-Zaitlik T. Bioinformatic and phenotypic characterization of bacteriophages encoded within and infecting *Klebsiella michiganensis*. MRes thesis, Nottingham Trent University. 2021.

Smith-Zaitlik T, Shibu P, McCartney AL *et al.* Extended genomic analyses of the broad-host-range phages vB_KmiM-2Di and vB_KmiM-4Dii reveal slopekviruses have highly conserved genomes. *Microbiology (Reading)* 2022;**168**, DOI: 10.1099/mic.0.001247.

Stock I, Wiedemann B. Natural antibiotic susceptibility of *Enterobacter amnigenus*, *Enterobacter cancerogenus*, *Enterobacter gergoviae* and *Enterobacter sakazakii* strains. *Clin Microbiol Infect* 2002;**8**:564–78.

Suescun-Sepulveda JA, Rondón González F, Fuentes Lorenzo JL. Diversity of culturable bacteria of freshwater environments from an altitudinal gradient in the eastern Cordillera of Colombia. *FEMS Microbiol Lett* 2023;**370**:fnad037.

Thakur P, Gauba P. Genomic characterization of *Lelliottia amnigena* PTJIIT1005, a nitrate tolerant strain isolated from water sample of Yamuna River, Delhi, India. *Microbiol Resour Announc* 2022;**11**:e0022922.

Tran PN, Md Zoqratt MZH, Michalczyk A *et al.* Genome sequence of *Lelliottia* sp. strain WAP21, isolated from soil in canola fields in Victoria, Australia. *Microbiol Resour Announc* 2022;**11**:e0101821.

Wiktorczyk-Kapischke N, Skowron K, Kwiecińska-Piróg J *et al.* Flies as a potential vector of selected alert pathogens in a hospital environment. *Int J Environ Health Res* 2022;**32**:1868–87.

Wu W, Zong Z. Genome analysis-based reclassification of *Lelliottia aquatilis* as a later heterotypic synonym of Lelliottia jeotgali. *Int J Syst Evol Microbiol* 2019;**69**:998–1000.

Yuk K-J, Kim Y-T, Huh C-S *et al. Lelliottia jeotgali* sp. nov., isolated from a traditional Korean fermented clam. *Int J Syst Evol Microbiol* 2018;**68**:1725–31.

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, 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, South Korea	4603334	1	54.2	4603334	4237	100	0.01
LST-1	CP063663	*Stevia*, 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, China	4361353	909	53.0	5972	4272	90.45	4.58
ERR1430553*	GCA_905202905	Human faeces, 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), 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, 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.

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

Strain	Accession	NCBI ID	rMLST ID, % support	ANI 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 %

423 *MAGs; full names ERR1430553_bin.131_CONCOCT_v1.1_MAG, ERR1430553-bin.48 and
424 ERR5094855_bin.4_metaWRAP_v1.3_MAG.
425

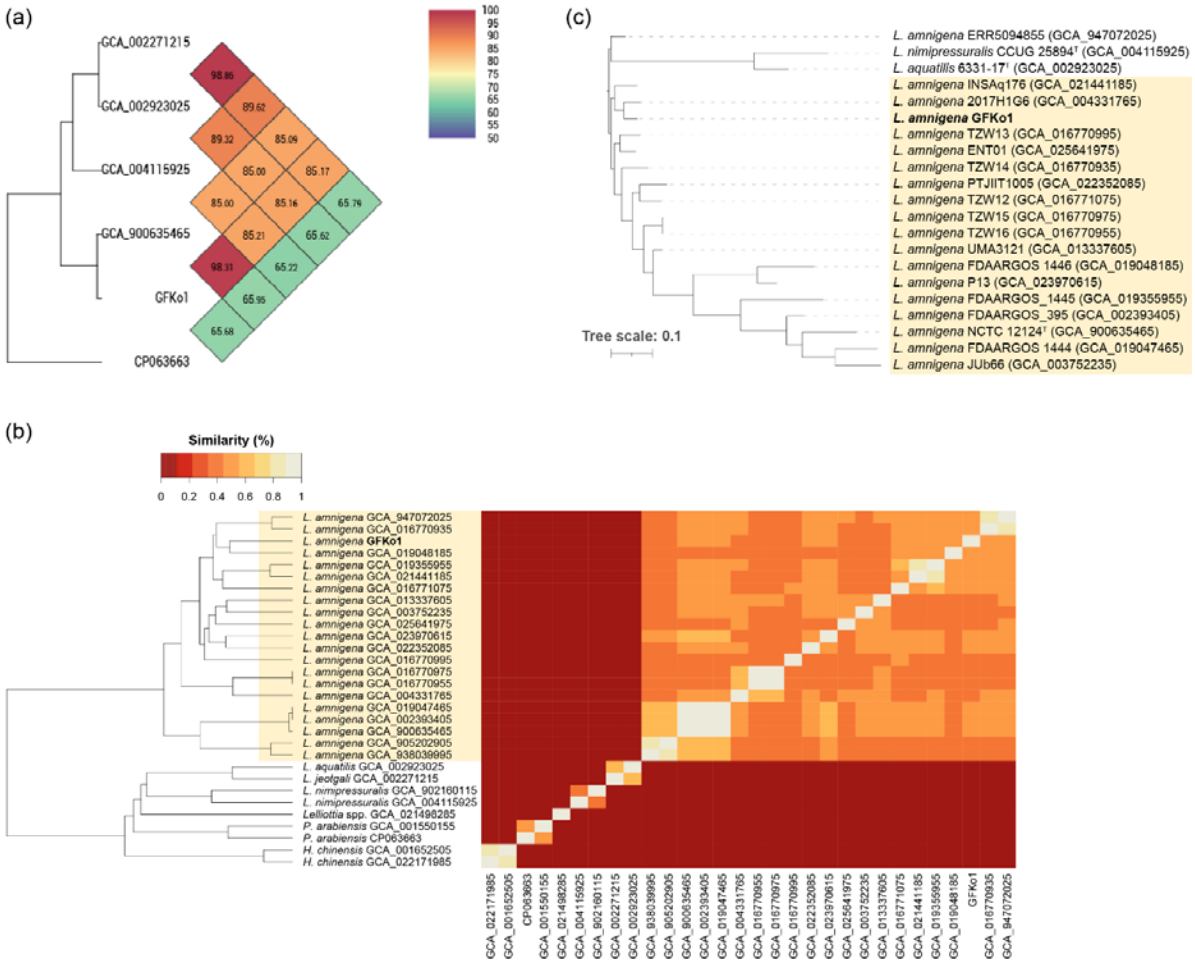


Fig. 1. Strain GFKo1 is a representative of *L. amnigena*. (a) Heatmap generated by OAT:OrthoANI showing the ANI between GFKo1 and strains listed as type strains of *Lelliottia* species with valid and non-valid names. GFKo1 shares highest ANI (%) with the type strain of *L. amnigena* (accession assembly 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*.

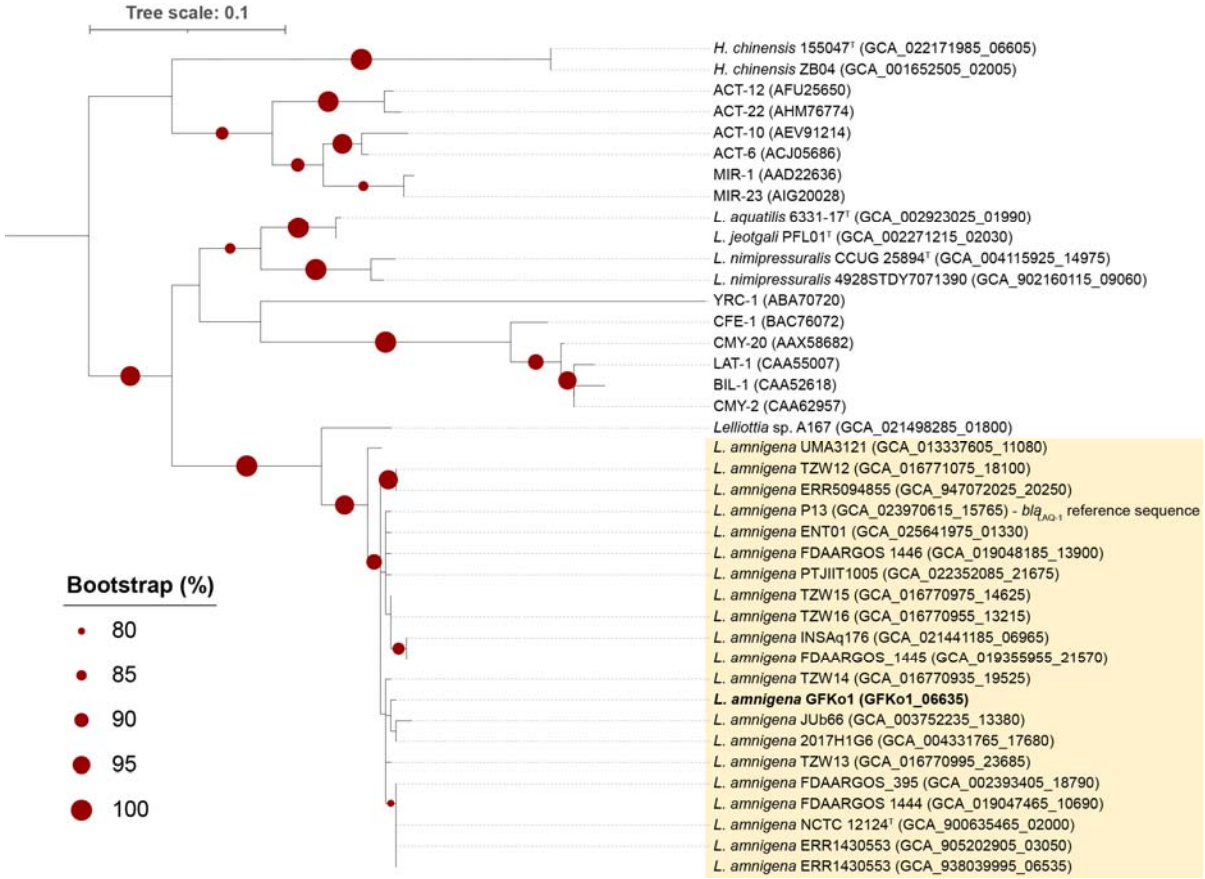
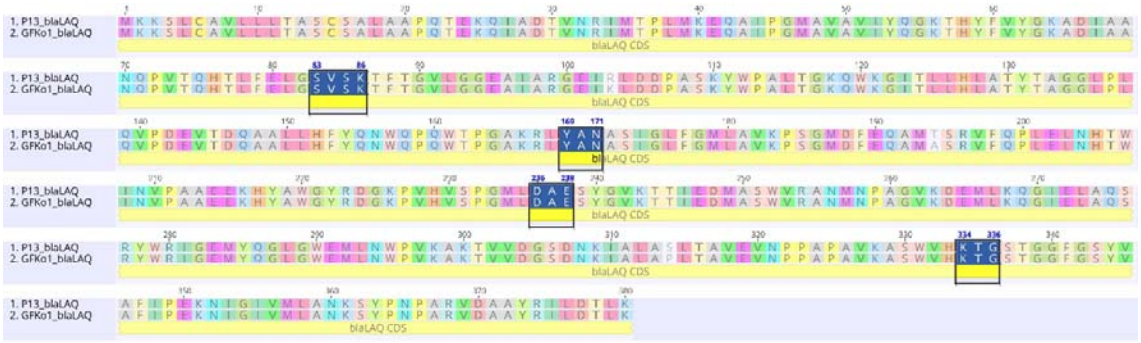


Fig. 2. *bla*$_{LAQ}$ is a core gene of *L. amnigena*. The *bla*$_{LAQ-1}$ sequence of *L. amnigena* P13 represents the reference for this chromosomally-encoded AmpC β-lactamase (Li *et al.* 2022). Twelve other AmpC β-lactamases (ACT-12, ACT-22, BIL-1, CMY-2, CMY-20, LAT-1, CFE-1, YRC-1, MIR-1, MIR-23, ACT-6, ACT-10; (Li *et al.* 2022)) 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 as **Supplementary Material**.



Supplementary Fig 1. Amino acid alignment of β-lactamase LAQ-1 from *L. amnigena* P13 (Accession QXM27670) with β-lactamase LAQ-1 from *L. amnigena* GFko1 (locus tag GFKo1_06635). Conserved amino acid sequences typical for a class C β-lactamase are highlighted in blue.

Supplementary Fig. 2. Alignment of *bla*$_{LAQ-1}$-like and surrounding genome region of strain GFKo1 with the same region of *L. amnigena* P13. Gene predictions and annotations were made using Bakta as described in Methods. The sequence of GFKo1 was reverse-complemented and the MAFFT alignment shown was created in Geneious Prime v2023.0.1. The alignment covers 21,897 bp; the sequences share 21,497 identical sites (97.6 % pairwise identity) at the nucleotide level. The region shown matches that analysed by (Li *et al.* 2022); their analysis included sequence data from *L. amnigena* strains P13, NCTC 12124^{T}, FDAARGOS 1444, FDAARGOS 1446, FDAARGOS_1445 and FDAARGOS_395.