**SUPPLEMENTARY MATERIAL ASSOCIATED WITH SHIBU *ET AL.***

# Shibu P, McCuaig F, McCartney AL, Kujawska M, Hall LJ, Hoyles L. Improved molecular characterization of the *Klebsiella oxytoca* complex reveals the prevalence of the kleboxymycin biosynthetic gene cluster.

**METHODS**

**Phenotypic characterization of clinical isolates.** Once in the laboratory, API 20E strips (bioMerieux) were used to confirm the identities of the clinical isolates as *K. oxytoca*, following manufacturer’s instructions.

**Antimicrobial susceptibility testing.** The three isolates were screened on Mueller–Hinton agar for possible carbapenemase production following UK national guidelines (1). This involved testing all presumptive isolates against 18 antimicrobials (amikacin, amoxycillin, augmentin, aztreonam, cefotaxime, cefoxitin, ceftazidime, cefuroxime, ciprofloxacin, colistin, ertapenem, gentamicin, meropenem, tazocin, temocillin, tigecycline, tobramycin, trimethoprim) following the EUCAST disc diffusion method. Control organisms used to monitor test performance of the antimicrobials were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213. Zone sizes were read using calibrated callipers and interpreted as sensitive or resistant by referring to the EUCAST breakpoint guidelines (2).

Carbapenemase confirmatory tests were performed on all three isolates as they were found to be resistant to the indicator carbapenem ertapenem. The isolates were further tested with ertapenem E-strips (Launch) to ascertain the Minimum Inhibitory Concentration (MIC). After incubation for 24 h at 30–35 °C the MIC gradients were read against the EUCAST breakpoint guidelines and those showing MICs >0.12 µg/mL were considered resistant to ertapenem (2).

**Extraction of DNA.** Isolates were plated onto MacConkey agar no. 3 (Oxoid) and incubated aerobically and overnight at 37 °C. On three separate passages, a single colony of each isolate was picked and streaked out. After the third passage, DNA was extracted from a loopful of cells using the Gentra PureGene Qiagen DNA extraction kit (Qiagen). DNA quality was assessed by agarose gel electrophoresis, and quantified using the Nanodrop instrument.

**Whole-genome sequencing, assembly and annotation.** Extracted DNA was frozen at -20 °C and sent to the Quadram Institute Bioscience, Norwich for library preparation and sequencing. Samples were run on an Illumina Nextseq500 instrument using a Mid Output Flowcell [NSQ® 500 Mid Output KT v2 (300 CYS)] following Illumina’s recommended denaturation and loading procedures, which included a 1% PhiX spike-in (PhiX Control v3). Data were uploaded to Basespace, where the raw data were converted to eight fastq files for each sample (four for R1, four for R2), which were subsequently concatenated to produce one R1 and one R2 file per strain. Sequence data were quality checked using fastqc v0.11.8 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>): no adaptor or over-represented sequences were present. Data were trimmed using trimmomatic 0.39 (SLIDINGWINDOW:5:20 MINLEN:50) (3), and paired reads retained. Genomes were assembled using the trimmed paired reads with SPAdes v3.13.0 (default settings) (4). Completeness and contamination of the three genomes was assessed using CheckM v1.0.18 (5). Gene predictions and annotations were completed using Prokka v1.14.5 (default settings) (6). The data have been deposited with links to BioProject accession number [PRJNA562720](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA562720) in the NCBI BioProject database, and under accession numbers [VTQC00000000](https://www.ncbi.nlm.nih.gov/nuccore/VTQC00000000), [VTQB00000000](https://www.ncbi.nlm.nih.gov/nuccore/VTQB00000000) and [VTQA00000000](https://www.ncbi.nlm.nih.gov/nuccore/VTQA00000000).

**Identification of antimicrobial and virulence genes.** The three draft genomes were uploaded to the *Klebsiella oxytoca* MLST website (<https://pubmlst.org/koxytoca/>) hosted by the University of Oxford (7) to determine allele number against previously defined house-keeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*). Kleborate (8,9) and Kaptive (<http://kaptive.holtlab.net>) were used to attempt to identify capsular (K) and O antigen types (8–10). Presence of antibiotic-resistance genes within strains was determined by BLASTP analysis of amino acid sequences of predicted genes within genomes against the Comprehensive Antibiotic Resistance Database (CARD) database v3.0.7 (downloaded 27 July 2019; protein homolog dataset) (11); only strict and perfect matches with respect to CARD database coverage and bit-score cut-off recommendations are reported, to reduce the potential for reporting false-positive results. Virulence genes were identified by BLASTP of genome amino acid sequences against the Virulence Factors of Pathogenic Bacteria Database (VFDB; ‘core dataset’ downloaded 27 July 2019) (12); results are reported for ≥70 % identity and 90 % query coverage (13).

***bla*OXY analysis.** *bla*OXY protein sequences available from the Institut Pasteur MLST and Whole Genome MLST Databases were [downloaded](https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef&page=downloadAlleles) on 6 February 2021. They were used to create a BLASTP database against which Prodigal-annotated (Prodigal v2.6.2; (14)) *K. oxytoca* complex genes were searched. *bla*OXY protein sequences were used to create a MSA (CLUSTAL W, BLOSUM matrix), from which a neighbour-joining tree was generated.

**Phylogenetic placement of the isolates within the *K. oxytoca* complex.** PhyloPhlAn v0.99 (15) was used to determine phylogenetic placements of the isolates within the *K. oxytoca* complex. PhyloPhlAn identifies hundreds of conserved (core) proteins from a given genomic dataset and uses them to build a complete high-resolution phylogeny.

**RESULTS**

**Antimicrobial susceptibility of the isolates**

PS\_Koxy1, PS\_Koxy2 and PS\_Koxy4 were shown to be isolates of *K. oxytoca* by phenotypic testing (API 20E profile 5245773, 97.8 % identity). The strains were all found to be resistant to amoxicillin (zone diameter <14 mm), augmentin (<19 mm), aztreonam (<21 mm), cefotaxime (<17 mm), cefoxitin (<19 mm), ceftazidime (<19 mm), cefuroxime (<18 mm), ciprofloxacin (<19 mm), ertapenem (<22 mm), gentamicin (<14 mm), tazocin (<17 mm), temocillin (<19 mm), tobramycin (<14 mm) and trimethoprim (<14 mm), and sensitive to amikacin (>18 mm), colistin (MIC <2 μg/mL), meropenem (>22 mm) and tigecycline (>18 mm). Ertapenem resistance was confirmed by E-test (had an MIC > 0.12 μg/mL), as ertapenem and meropenem are used as an indicator antibiotic for the detection of carbapenemase.

**Genotypic characterization of the three clinical isolates**

We generated draft genome sequence data for PS\_Koxy1, PS\_Koxy2 and PS\_Koxy4 to accurately identify the strains and provide us with genomic data that would be useful in our future phage studies (**Supplementary** **Table A**).

**Supplementary Table A.** Sequencing summary statistics for the three clinical isolates characterized in this study (all 60× coverage)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Isolate** | **Size (bp)** | **No. contigs** | **N50** | **CDS** | **Completeness, contamination**\* |
| PS\_Koxy1 | 6,271,778 | 135 | 170,962 | 5,948 | 100 %, 0.30 % |
| PS\_Koxy2 | 6,275,379 | 111 | 172,981 | 5,946 | 100 %, 0.30 % |
| PS\_Koxy4 | 6,294,880 | 112 | 146,343 | 5,978 | 100 %, 0.30 % |

\*Determined using CheckM v1.0.18.

**Antimicrobial resistance (AMR) gene profiles of the three clinical isolates**

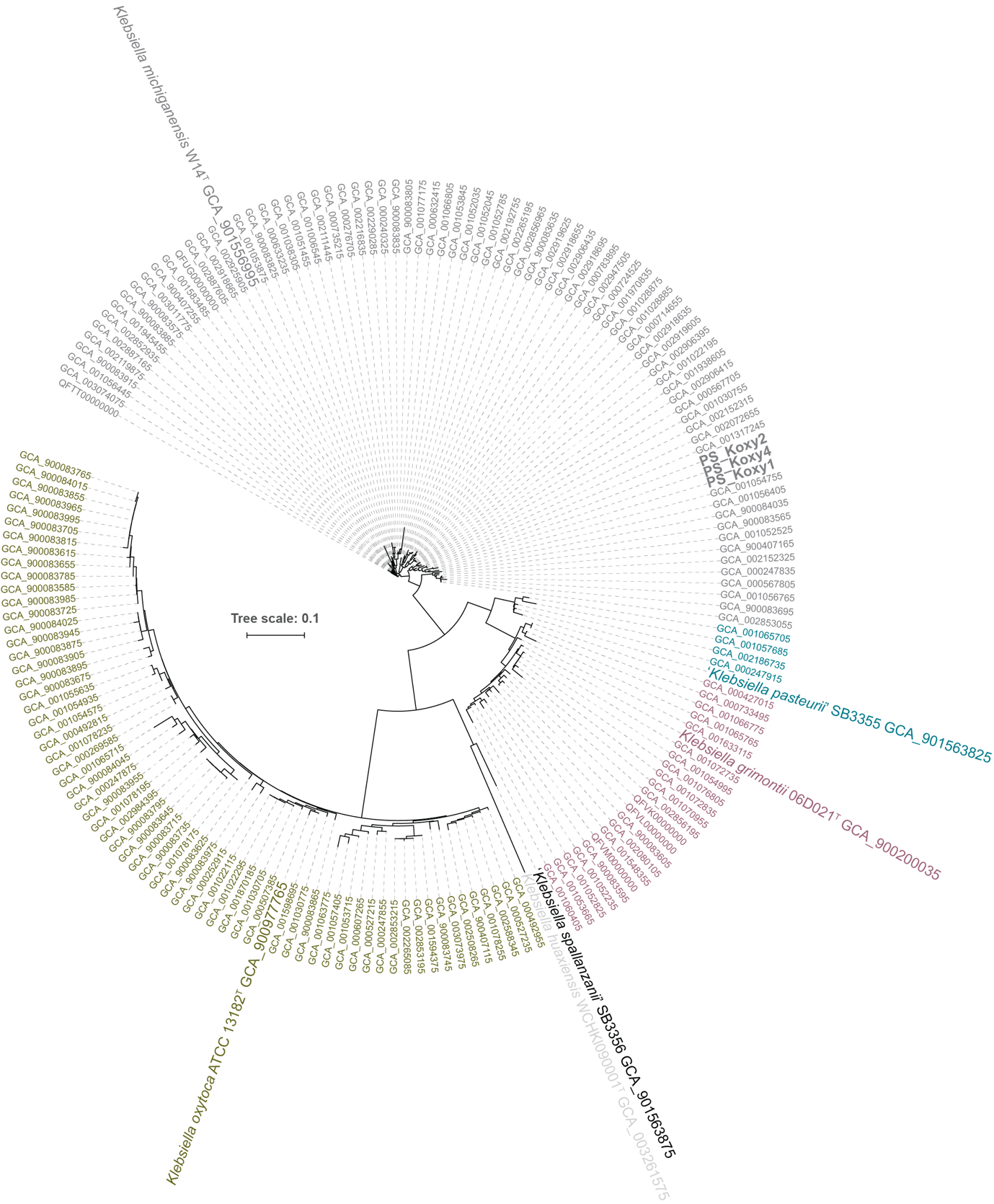
All three isolates encoded *bla*OXY1-8, showing they belonged to *K. michiganensis*, not *K. oxytoca* (**Supplementary Figure 1**). Phylogenetic analyses of the isolates with representatives of the *K. oxytoca* complex confirmed their affiliation with *K. michiganensis* (**Supplementary Figure 2**). The protein sequences encoded by the strains’ genomes were compared against the CARD database (11). Presence of the β-lactamase with carbapenemase activity GES-5 (100 % identity, bit-score 591 – perfect CARD match) was confirmed, so too was that of the extended spectrum β-lactamase (ESBL) CTX-M-15 (100 % identity, bit-score 593 – perfect CARD match) (**Supplementary Figure 3**) (16). PS\_Koxy1, PS\_Koxy2 and PS\_Koxy4 also encoded SHV-66 (99.65 % identity, bit-score 580 – strict CARD match). In addition to the β-lactamases GES-5, CTX-M-15 and SHV-66, PS\_Koxy1, PS\_Koxy2 and PS\_Koxy4 encoded several other antibiotic-resistance genes (**Supplementary Figure 3a**), some reported as rare (e.g. *acrB*, *acrD*, *emrB*, *mdtB*, *mdtC*) in *K. oxytoca* genomes by CARD while others were common (e.g. *baeR*, *fosA5*, CRP, *marA*) (**Supplementary** **Table B**). Furthermore, PS\_Koxy1 encoded AAC(6’)-Ib7; PS\_Koxy1 and PS\_Koxy2 encoded *tet*(*A*); PS\_Koxy2 and PS\_Koxy4 encoded QnrB1 (**Supplementary** **Figure 3a**).

PS\_Koxy1, PS\_Koxy2 and PS\_Koxy4 were resistant to the β-lactam antibiotics amoxicillin and aztreonam, augmentin and tazocin (both containing a β-lactam antibiotic and β-lactamase inhibitor), the cephalosporins cefotaxime, cefoxitin, ceftazidime and cefuroxime, the fluoroquinolone ciprofloxacin, the carbapenem ertapenem, the aminoglycosides gentamicin and tobramycin, the carboxypenicillin temocillin, and the antifolate antibacterial trimethoprim. They were sensitive to amikacin (aminoglycoside), colistin (polymyxin), meropenem (carbapenem) and tigecycline (glycylcycline). *bla*OXA-1 is frequently associated with *bla*CTX-M-15, making isolates resistant to β-lactam–β-lactamase inhibitor combinations (19): all three strains were resistant to augmentin (amoxicillin/clavulanic potassium) and tazocin (piperacillin/tazobactam), with neither OXA-1 nor CTX-M-15 considered common in *K. oxytoca* genomes (**Supplementary Figure 3b**, **Supplementary Table B**). The strains carried a range of plasmid-encoded enzymes [AAC(6’)-Ib7, *aadA,* APH(3’’)-Ib, APH(6)-Id, AAC(3)-IIe] conferring resistance to gentamicin and tobramycin, though they remained sensitive to amikacin.

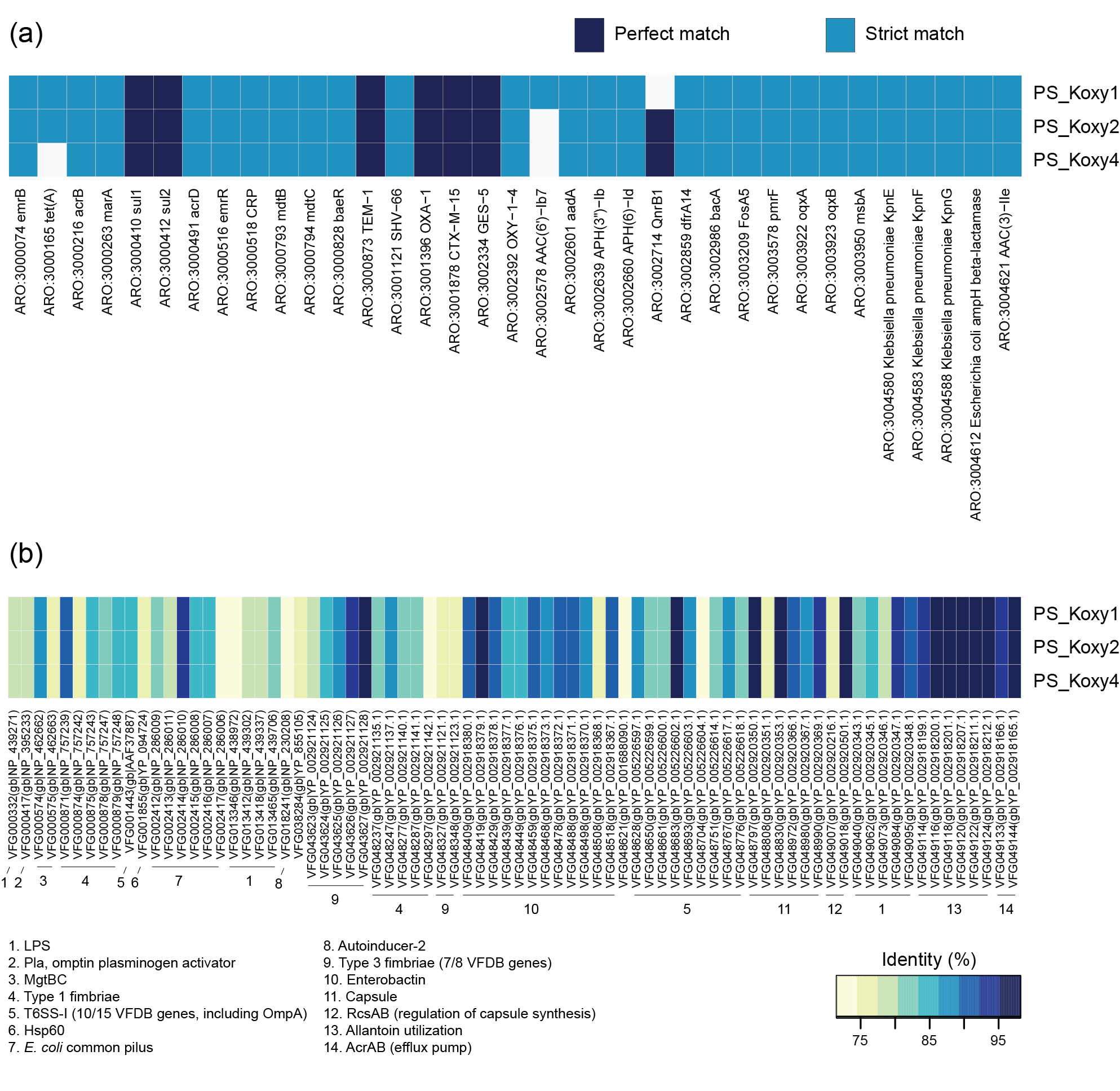
Chart, sunburst chart

Description automatically generated

**Supplementary Figure 1.** *bla*OXY gene analysis shows PS\_Koxy1, PS\_Koxy2 and PS\_Koxy4 (in white text) belong to phylogroup Ko1 and, therefore, are affiliated with *K. michiganensis*. The phylogenetic tree (neighbour joining, Jukes Cantor) was generated using *bla*OXY-encoding protein sequences from the genomes of the three clinical isolates and publicly available *K. oxytoca* complex genomes (**Supplementary Table 1**). Scale bar, average number of amino acid substitutions per position.

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**Supplementary Figure 2.** Phylogenetic tree showing the relationship of the three GES-5-positive clinical isolates (PS\_Koxy1, PS\_Koxy2, PS\_Koxy4) and the 167 strains included in our previous study (13), plus reference strains of species of the *K. oxytoca* complex (17). The tree was generated using PhyloPhlAn v0.99 and 380 protein-encoding sequences conserved across all genomes. Scale bar, average number of amino acid substitutions per position.



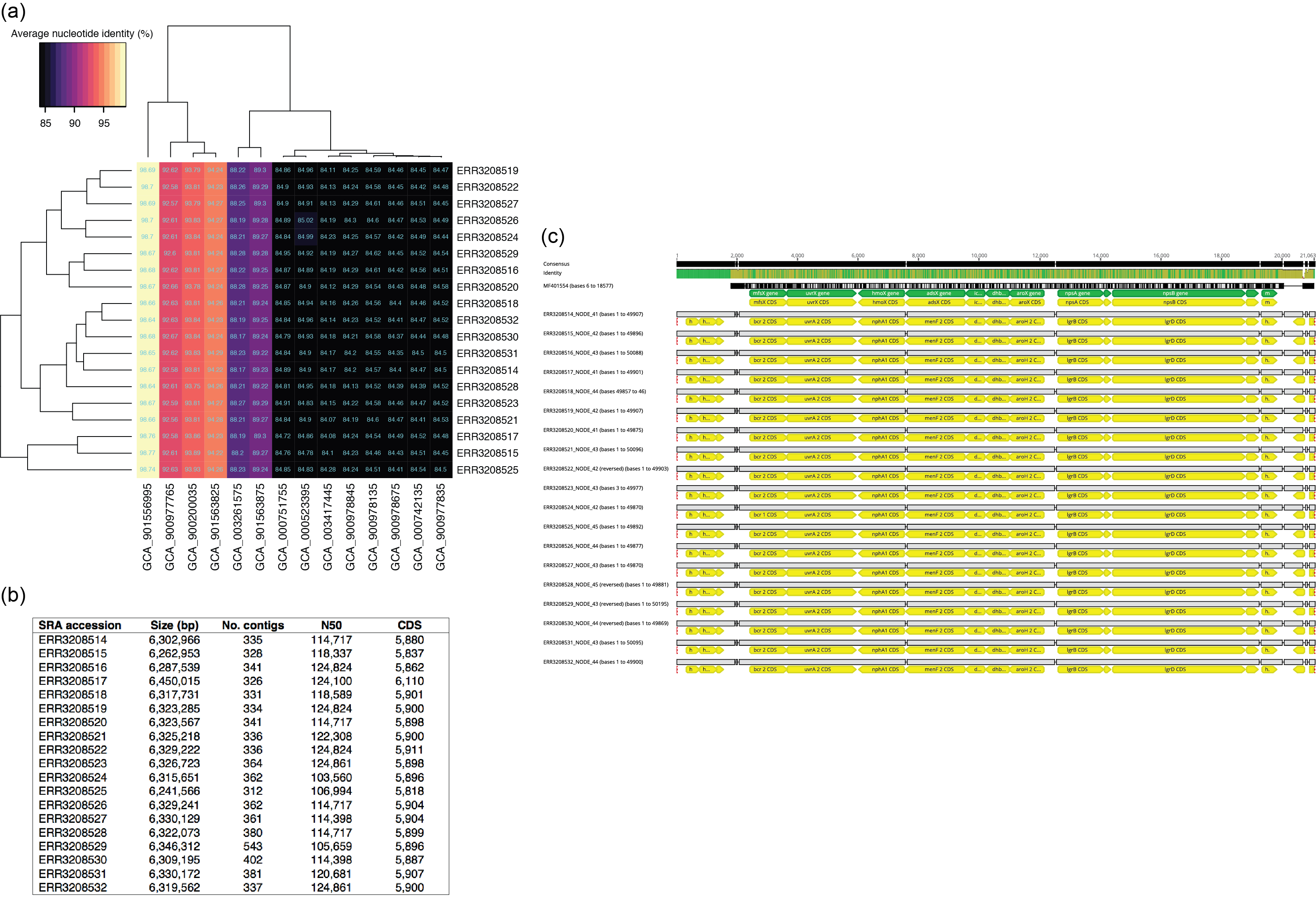
**Supplementary Figure 3.** Antimicrobial resistance (a) and virulence factor (b) genes detected in the genomes of the GES-5-positive *K. michiganensis* strains. (a) Protein sequences encoded within the strains’ genomes were compared against sequences in CARD (11). Strict CARD match, not identical but the bit-score of the matched sequence is greater than the curated BLASTP bit-score cut-off; perfect CARD match, 100 % identical to the reference sequence along its entire length. (b) Protein sequences encoded within the strains’ genomes were compared against sequences in VFDB (12). Only BLASTP results for proteins sharing >70 % identity and 90 % query coverage with VFDB protein sequences are shown.

**Supplementary Table B.** Summary of information for CARD genes found in *K. michiganensis* PS\_Koxy1, PS\_Koxy2 and PS\_Koxy4

CARD data from analyses of 257 *K. oxytoca* complex genomes (<https://card.mcmaster.ca/prevalence>). CARD Prevalence 3.0.7 is based on sequence data acquired from NCBI on 7 May 2020.

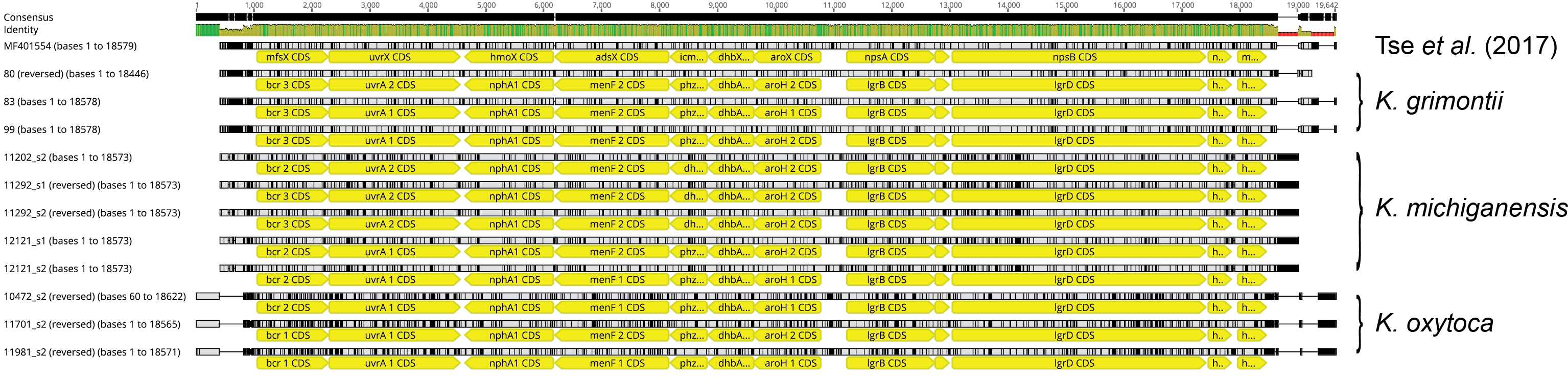
| **ARO: accession** | **Name** | **Definition** | **Prevalence (%) in *K. oxytoca*\*** |
| --- | --- | --- | --- |
| 3000074 | *emrB* | Translocase in the *emrB*-TolC efflux protein in *E. coli*. It recognises substrates including carbonyl cyanide *m*-chlorophenylhydrazone, nalidixic acid and thioloactomycin. | 0.78 |
| 3000165 | *tet*(*A*) | Tetracycline efflux pump found in many species of Gram-negative bacteria. | 5.84 |
| 3000216 | *acrB* | Protein subunit of AcrA-AcrB-TolC multidrug efflux complex. AcrB functions as a heterotrimer which forms the inner membrane component and is primarily responsible for substrate recognition and energy transduction by acting as a drug/proton antiporter. | 0.78 |
| 3000263 | *marA* | In the presence of antibiotic stress, *E. coli* overexpresses the global activator protein MarA, which besides inducing MDR efflux pump AcrAB, also down-regulates synthesis of the porin OmpF. | 98.05 |
| 3000410 | *sul1* | Sulfonamide resistant dihydropteroate synthase of Gram-negative bacteria. It is linked to other resistance genes of class 1 integrons. | 13.23 |
| 3000412 | *sul2* | Sulfonamide resistant dihydropteroate synthase of Gram-negative bacteria, usually found on small plasmids. | 2.72 |
| 3000491 | *acrD* | Aminoglycoside efflux pump expressed in *E. coli*. Its expression can be induced by indole, and is regulated by *baeRS* and *cpxAR*. | 0.78 |
| 3000516 | *emrR* | EmrR is a negative regulator for the EmrAB-TolC multidrug efflux pump in *E. coli*. Mutations lead to EmrAB-TolC overexpression. | 99.61 |
| 3000518 | CRP | CRP is a global regulator that represses MdtEF multidrug efflux pump expression. | 100 |
| 3000793 | *mdtB* | MdtB is a transporter that forms a heteromultimer complex with MdtC to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex. | 0.39 |
| 3000794 | *mdtC* | MdtC is a transporter that forms a heteromultimer complex with MdtB to form a multidrug transporter. MdtBC is part of the MdtABC-TolC efflux complex. In the absence of MdtB, MdtC can form a homomultimer complex that results in a functioning efflux complex with a narrower drug specificity. | 0.39 |
| 3000828 | *baeR* | BaeR is a response regulator that promotes the expression of MdtABC and AcrD efflux complexes. | 99.22 |
| 3000873 | TEM-1 | TEM-1 is a broad-spectrum beta-lactamase found in many Gram-negative bacteria. Confers resistance to penicillins and first generation cephalosphorins. | 5.45 |
| 3001121 | SHV-66 | SHV-66 is an extended-spectrum beta-lactamase found in *K. pneumoniae*. | ND\* |
| 3001396 | OXA-1 | OXA-1 is a beta-lactamase found in *E. coli*. | 1.95 |
| 3001878 | CTX-M-15 | CTX-M-15 is a beta-lactamase found in the family *Enterobacteriaceae*. | 0.78 |
| 3002334 | GES-5 | GES-5 is a beta-lactamase found in the family *Enterobacteriaceae*. | ND |
| 3002392 | OXY-1-4 | OXY-1-4 is a beta-lactamase found in *K. oxytoca*. | 3.11 |
| 3002578 | AAC(6’)-Ib7 | AAC(6')-Ib7 is a plasmid-encoded aminoglycoside acetyltransferase in *Enterobacter cloacae* and *Citrobacter freundii*. | ND |
| 3002601 | *aadA* | ANT(3'')-Ia is an aminoglycoside nucleotidyltransferase gene encoded by plasmids, transposons, integrons in *Enterobacteriaceae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Vibrio cholerae*. | 8.95 |
| 3002639 | APH(3’’)-Ib | APH(3'')-Ib is an aminoglycoside phosphotransferase encoded by plasmids, transposons, integrative conjugative elements and chromosomes in *Enterobacteriaceae* and *Pseudomonas* spp. | 4.67 |
| 3002660 | APH(6)-Id | APH(6)-Id is an aminoglycoside phosphotransferase encoded by plasmids, integrative conjugative elements and chromosomal genomic islands in *K. pneumoniae*, *Salmonella* spp., *E. coli*, *Shigella flexneri*, *Providencia alcalifaciens*, *Pseudomonas* spp., *V. cholerae*, *Edwardsiella tarda*, *Pasteurella multocida* and *Aeromonas bestiarum.* | 5.45 |
| 3002714 | QnrB1 | Plasmid-mediated quinolone resistance protein found in *K. pneumoniae*. | 0.39 |
| 3002859 | *dfrA14* | Integron-encoded dihydrofolate reductase found in *E. coli.* | 5.45 |
| 3002986 | *bacA* | BacA recycles undecaprenyl pyrophosphate during cell wall biosynthesis, which confers resistance to bacitracin. | 0.39 |
| 3003209 | *fosA5* | Fosfomycin resistance gene isolated from clinical strain of *E. coli* E265. It is susceptible to amikacin, tetracycline and imipenem, and resistant to sulphonamide, cephalosporins, gentamicin, ciprofloxacin, chloramphenicol and streptomycin. | 93.39 |
| 3003578 | *pmrF* | Required for the synthesis and transfer of 4-amino-4-deoxy-L-arabinose to Lipid A, which allows Gram-negative bacteria to resist the antimicrobial activity of cationic antimicrobial peptides and antibiotics such as polymyxin. | 0.39 |
| 3003922 | *oqxA* | RND efflux pump conferring resistance to fluoroquinolone. | 91.83 |
| 3003923 | *oqxB* | RND efflux pump conferring resistance to fluoroquinolone. | 1.56 |
| 3003950 | *msbA* | Multidrug resistance transporter homolog from *E. coli* and belongs to a superfamily of transporters that contain an adenosine triphosphate (ATP) binding cassette (ABC) which is also called a nucleotide-binding domain (NBD). MsbA is a member of the MDR-ABC transporter group by sequence homology. MsbA transports lipid A, a major component of the bacterial outer cell membrane, and is the only bacterial ABC transporter that is essential for cell viability. | 98.83 |
| 3004580 | *K. pneumoniae* KpnE | KpnE subunit of KpnEF resembles EbrAB from *E. coli*. Mutation in KpnEF resulted in increased susceptibility to cefepime, ceftriaxon, colistin, erythromycin, rifampin, tetracycline, and streptomycin as well as enhanced sensitivity toward sodium dodecyl sulfate, deoxycholate, dyes, benzalkonium chloride, chlorhexidine and triclosan. | 98.83 |
| 3004583 | *K. pneumoniae* KpnF | KpnF subunit of KpnEF resembles EbrAB from *E. coli*. Mutation in KpnEF resulted in increased susceptibility to cefepime, ceftriaxon, colistin, erythromycin, rifampin, tetracycline, and streptomycin as well as enhanced sensitivity toward sodium dodecyl sulfate, deoxycholate, dyes, benzalkonium chloride, chlorhexidine and triclosan. | 99.22 |
| 3004588 | *K. pneumoniae* KpnG | KpnG consists of ~390 residues and resembles EmrA of *E. coli*. Disruption of the pump components KpnG-KpnH significantly decrease resistance to azithromycin, ceftazidime, ciprofloxacin, ertapenem, erythromycin, gentamicin, imipenem, ticarcillin, norfloxacin, polymyxin-B, piperacillin, spectinomycin, tobramycin and streptomycin. | 99.22 |
| 3004612 | *E. coli ampH* | AmpH is a class C ampC-like beta-lactamase and penicillin-binding protein identified in *E. coli*. | 99.22 |
| 3004621 | AAC(3)-IIe | Plasmid-encoded aminoglycoside acetyltransferase in *E. coli*. | 1.95 |

\*ND, no data.



**Supplementary Figure 4.** Species identification of ST138 isolates described by Ellington *et al.* (18) and detection of the kleboxymycin BGC within their genomes. (a) Bidirectional clustered heatmap showing that all ST138 isolates described recently are *K. michiganensis*, not *K. oxytoca*, sharing 98.64–98.77 % ANI withGCA\_901556995 (*K. michiganensis* W14T). (b) Assembly statistics for the genomes assembled in this study with the Sequence Read Archive (SRA) accession numbers associated with the raw data. (c) The kleboxymycin BGC is present in all 19 of the *K. michiganensis* ST138 isolates of Ellington *et al.* (18). The image (alignment view) was generated via the progressiveMauve algorithm plugin of Geneious Prime v2019.2.1 (default settings, full alignment). Prokka-assigned gene annotations have been left for the *de novo*-assembled genomes. Consensus identity is the mean pairwise nucleotide identity over all pairs in the column: green, 100 % identity; greeny-brown, at least 30 % and under 100 % identity; red, below 30 % identity.

ERR3208533 was also assembled but was found to be *K. oxytoca* and lacking the kleboxymycin BGC (data not shown, but assembly available from [figshare](http://doi.org/10.6084/m9.figshare.11923014)).



**Supplementary Figure 5.** Detection of the kleboxymycin BGC in strains and MAGs recovered from the faecal microbiota of preterm infants. The strains and MAGs were characterized by us previously (13). The image (alignment view) was generated via the progressiveMauve algorithm plugin of Geneious Prime v2019.2.1 (default settings, full alignment). Prokka-assigned gene annotations have been left for the infant-associated strains and MAGs. Two pairs of the *K. michiganensis* MAGs came from the same infants (11292, 12121) sampled at two different time points. Consensus identity is the mean pairwise nucleotide identity over all pairs in the column: green, 100 % identity; greeny-brown, at least 30 % and under 100 % identity; red, below 30 % identity.

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