Isolation, identification, and genomic characterization of bacterial isolate FO1-S19 from American Burying beetle *Nicrophorus americanus*

Sarah R. Yanchick

Oklahoma State University

May 2022

INTRODUCTION

The American burying beetle, *Nicrophorus americanus,* also known as giant carrion beetle is a carnivorous endangered carrion species that once were found throughout 35 different states in the United States. Current data suggest they can live in a wide variety of habitats, however, they do require specifically sized carrions (e.g. chipmunks and doves) in order to successfully reproduce. Their shiny black body with striking orange-red tinted markings are distinctive characteristics of the species, and their unique means of bearing brood is what sets these beetle species apart from other beetles. During their mating season, once a year, in June or July, female and male beetles search for animal carcasses (carrion) that will be utilized by their offspring (Khetani et. al., 2011). After mating atop the carrion, the beetles begin chewing, burying, and most notably, covering the carcass with antimicrobial oral and anal secretions to form a brood ball (Carrie et. al., 2011). The female will then lay her eggs within the brood ball and once the larvae hatch, they have an ample source of food available from the ball until they metamorphosize into adults (Khetani et. al., 2011).

Of the most interesting aspects of the oral and anal secretions of *Nicrophorus* species' is that they may harbor antimicrobial properties. The working hypothesis is that these secretions inhibit microbes that would normally cause the flesh to putrefy and essentially "mummify" the carrion while retaining its nutritional value for the larvae (Pratt 2018). Several studies have found evidence of antimicrobial peptides (Rana et al., 1997), metabolites (Degenkolb et. al., 2011), and lysozymes (Jacobs et. al., 2016) within the secretions of *Nicrophorus spp*. Degenkolb et. al., (2011) characterized secondary metabolites from *Nicrophorus vespilloides* and reported the presence of phenol, p-cresol, indole, 2-phenylethyl alcohol, benzoic acid pelargonic acid, phenylactic acid, quinoline, 3-phenyl-propionic acid, p-hydroxy-benzoic acid methyl ester, p-hydroxy-phenylacetic

acid, methyl myristate, myristic acid, methylpalmitate, palmitic acid, methyl stearate, and caprylic acid. All these compounds have known antimicrobial effects and are hypothesized as contributors to Nicrophorus spp. anti-putrefactive and anti-microbial characteristics (Degenkolb et. al., 2011) Metabolites, antimicrobial peptides, and lysosomes are associated with the antimicrobial characteristic of *Nicrophorus* secretions that have been reported on. However, it is not fully understood if these compounds are produced by the beetles themselves or are secondary metabolites from bacteria populations.

Our laboratory has previously performed a pilot study in which, oral and anal secretions from *N. americanus* were screened for aerobic microbes. From thie pilot study, Pratt et. al., (2018) was able to identify the isolation of *Gordonia, Proteus, Acinetobacter, Myroides, Pseudochrobactrum, Corynebacterium, Vitreoscilla, Paracoccus, and Glutamicibacter* species from these secretions. Most of these organisms, if not all, can be found in environmental soil samples (Andalibi et, al., 2017, Drzewiecka 2016, Rahal et. al., 2000, Beharrysingh 2017, Kämpfer et. at., 2006, Bernard et. at., 2016, Pringsheim 1949, Euzéby 1997, Vogel et al., 2017, Puri et. al., 2022, Aalborg University). Soil bacteria face an intense, highly populated microbiome in soil and have thus evolved to naturally produce antibiotics to give themselves a better chance of survival to limit competition.

Acinetobacter spp. are ubiquitous and can be found in a myriad of habitats including soil, water, human skin, mucous membranes, vegetation, and sewage (Rahal et. al., 2000). The genus *Acinetobacter* are Gram-negative opportunistic pathogens that are associated with severe nosocomial infections including pneumonia, bloodstream, urinary tract, and wound infections, as well as meningitis (Abo-Zed et. al., 2020, Mohd, et., al., 2017). These organisms also harbor multiple antibiotic-resistant determinants which allows them to resist the action of almost every

antibiotic used to treat infections caused by these organisms (Fournier et. al., 2006). *Acinetobacter spp*. have previously been isolated from multiple insects including *Aedes albopictus* (Minard et. al., 2012), *Helicoverpa armigeras'* gut (Malhorta et. al., 2012), the Red Flower Beetle larva (Wang et. al., 2020), the teste fly midgut (Geiger et. al., 2009), and *Gryllotalpa africana* (Banerjee et. al., 2020).

Acinetobacter spp., particularly *Acinetobacter baumanii*, are of great concern in healthcare since they can cause serious infections recalcitrant to therapy because these bacterial pathogens often express a multiple antibiotic resistance, or multidrug resistance (MDR) phenotype (Ju et. al., 2021). These bacterial pathogens can also survive for long periods of time on surfaces (Manchanda et. al., 2010). Manchanda et. al., (2010) reported that the most prominent *Acinetobacter* species present on human skin and mucous membranes are *Acinetobacter lwoffi*, *Acinetobacter johnsonii,* and *Acinetobacter junii*. *A. baumanii* however is undeniably the most important *Acinetobacter* species in terms of nosocomial infections and was rarely found on human skin (Manchanda et. al., 2010). Because these pathogens express MDR, carbapenems are commonly used for the treatment of infections caused by these organisms. With the identification of the OXA-23 gene encoding carbapenemase in *Acinetobacter spp*., carbapenems may one day no longer demonstrate efficacy in treating infections caused by these organisms (Corrêa et. al., 2012).

For this honors thesis, one isolate, FO1-S19, which had been identified as *Acinetobacter* sp. by MALDI-TOF-MS, was selected to to further characterize this isolate and its phylogenetic relationship to other *Acinetobacter* species. We initially performed 16S rDNA sequencing to help identify the species and produced a draft genome of this isolate. The genome sequence was completed to identify genes encoding antimicrobial compounds and virulence genes. We also

intended to confirm the species of this organism and determine where it clades in regard to other *Acinetobacter* sp., by performing a phylogenetic analysis based on 16S rDNA sequences.

MATERIALS AND METHODS

All culturing was conducted under a biological safety hood using appropriate aseptic techniques. NA was prepared by combining 500 mL of deionized water, 4 grams of nutrient broth (Difco, USA), and 7.5 grams of granulated agar (Difco, USA). The mixture was thoroughly mixed and then autoclaved for 15 min (121°C), cooled for approximately 45-50 minutes, and then poured into sterile plastic petri dishes.The bacterial isolate FO1-S19 stock was revived and struck onto nutrient agar (NA) and incubated at 25°C for 2-3 days. A single isolated colony was then used to inoculate fresh nutrient broth (NB) which was incubated at 25°C while being rotated (200 rpm).

Genomic DNA (gDNA) of *FO1-S19* was isolated using a GenElute Bacterial genomic DNA Kit (Sigma-Aldrich, USA). To isolate total DNA from strain *FO1-S19,* initially a 1.0 mL overnight culture (37°C, 200 rpms), was pipetted into a 1.5 mL Eppendorf tube, and the cells were harvested by centrifugation (9000 rpm, 10 min). The supernatant was then disposed, and the cell pellet was resuspended in 1 mL TE buffer (Tris-EDTA, 8.0 pH), then centrifuged (9000 rpm, 10 min). Next, the cell pellet was resuspended in 500 mL of lysis buffer (1% SDS, 100 mM EDTA, and 50 mM Tris pH 8) and 5 µL of protein kinase K (20 µg/µL) and RNAse (100 µg/µL) was added to the tube. These suspensions were then mixed via inversion and incubated at 37º C for 1 hr in an incubator (37°C). After incubation, 1 mL of chloroform: isoamyl alcohol (24:1) was added and the solution was mixed well, and further incubated at 25°C. After 5 min, the mixture was centrifuged (9000 G, 10 min, 4ºC). The highly viscous supernatant was then

collected and transferred to a sterile 1.5 ml Eppendorf tube. The chloroform: isoamyl alcohol extraction process was then repeated. Next, 0.8 volume of isopropanol was added and mixed gently via inversion until white strands precipitated in the tube. The mixture was then incubated at 4º C for 30 min. to overnight, and the contents were then centrifuged (9000 G, 10 min) and the supernatant was removed. The pellet was then washed with 1 mL of 70% ethanol, and then centrifuged again (9000 G, 10 min). The aqueous layer was then removed, and the resulting pellet was air-dried at 25°C for 20-30 min. When dried, 50 µL of TE (Tris-EDTA Buffer, 8.0 pH) was added to the DNA pellet to dissolve it.

After isolation, 5-8 µL (20-50 ng) of the dissolved DNA was checked on 1.2% agarose gel for DNA integrity. Genomic DNA was run at 80V constant voltage for 30-45 min and gel was visualized on Gel-Doc (Bio-Rad, USA). DNA quantity and quality were then measured on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Twenty ng of the DNA was then subjected to 16S rDNA amplification using 16s rDNA universal primers.

The development of molecular techniques based on sequence variability in the 16S rRNA genes has led to an improved understanding of the microbial communities present in a variety of ecosystems, including the gut microbiota (Suau et. al., 1999) Primer 16SrRNA_27F-5`AGAGTTTGATCMTGGCTCAG-3` and 16SrRNA_1492R-5`GGTTACCTTGTTACGACTT-3` is the most widely used primer for species-level identification (Frank et. al., 2008). Nearly full-length 16S rRNA genes were amplified using the 16SrRNA_27F and 16SrRNA_1492r primer set.

Twenty ng of genomic DNA was subjected to 16s rDNA amplification using PCR or polymerase chain reaction methods. Each 20 µL PCR reaction mixture consisted of genomic

DNA (20 ng), 10 µL OneTaq® Quick-Load 2X Master Mix (NEB, USA), 1 µL forward primer (10 uM), and 1 µL reverse primer (10 uM). This mixture was then put into the BioRad MyCycler thermal cycler (Bio-Rad, USA). The cycle was set to run with the following cycling conditions: initial denaturation at 95°C for 5 min, 36 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 68°C for 1 min, and 30 sec. A final extension was performed at 72°C for 6 min. The whole process took approximately 3 hr. After the PCR was performed, 8 µL of the amplicon was run on 1.2% agarose gel through electrophoresis for 30-45 min at constant voltage (80 V) and gel was visualized on Gel-Doc (Bio-Rad, USA). PCR products were then diluted 5 times and 1 µL of the diluted amplicon was used as template for re-amplification using the PCR condition described previously. A fraction of the re-amplified product (5 µL) was checked on the agarose gel and remaining re-amplified product was purified using the PCR cleanup kit (NEB, USA) using standard protocol as per manufacturer's instructions. The purified PCR products were sequenced using the BigDye Terminator Sequencing Kit (Applied Biosystems, U.S.A.).

The obtained Sanger's sequencing data of 16S rRNA region were merged using CAP3 (Huang and Madan, 1999). The consensus sequences were subjected to nucleotide blast in nr database of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rDNA sequences of the relative species were retrieved from the NCBI database (blast.ncbi.nlm.nih.gov) and used for construction of phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou et. al., 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the

bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et. al., 2004) and are in the units of the number of base substitutions per site. This analysis involved 12 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1307 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et. al., 2018).

For genomic DNA library preparation the concentration of extracted DNA was calculated using a Qubit®double-stranded DNA (dsDNA) high-sensitivity (HS) Assay Kit (Life Technologies, Inc., Carlsbad, CA), and the integrity of the gDNA was determined using a NanoDrop™ spectrophotometer. Illumina sequencing libraries were prepared using a Nextera™ XT DNA Sample Preparation Kit and Nextera™ XT Index Kit (Illumina Inc., San Diego, CA). The Illumina library was quantified using a Qubit® DNA HS Assay Kit in a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA), and the size of the library was checked using an Agilent 2100 Bioanalyzer System with an Agilent HS DNA Kit (Agilent Technologies, Santa Clara, CA). The library was then sequenced on an Illumina MiniSeq platform (Illumina Inc., USA) using a MiniSeq high output reagent cartridge for 300 cycles. The paired-end reads were de novo assembled into contigs using an A5-MiSeq assembler (Coil et. al., 2015). The contigs were annotated using the PROKKA (Seemann 2014). Antimicrobial resistance genes, virulence genes, and in silico plasmid replicon types were predicted using ARG-ANNOT (Gupta et. al., 2014), VirulenceFinder (https://cge.cbs.dtu.dk/services/VirulenceFinder/) and PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) respectively under Abricate (https://github.com/tseemann/abricate). Lipoprotein signal peptides and numbers of

transmembrane helices were predicted using SignalP (Bendtsen et. al., 2004) and TMHMM (Krogh et. al., 2001), respectively. CGView was used for the visualization of circular genomes and features (Stothard et. al., 2005).

A secondary set of DNA amplification and isolation took place in this study involving 38 different unknown bacteria isolated from oral and anal secretion of *Nicrophorus americanus*. Swabs were streaked onto nutrient agar plates and individual colonies with varied colony morphologies were subsequently re-streaked until separate isolates were confirmed. All isolates were subjected to 16S rDNA amplification using colony PCR. Single isolated colonies from NA plates were picked and mixed in 50 µL of TET (Tris 1 mM, EDTA 10 mM, Triton X-100 0.2%) Buffer. The cells were lysed at 99°C for 10 min in the MyCycler thermal cycler (Bio-Rad, USA). Lysed cells were spin briefly to settle to cell debris and supernatant was then used as template for the amplification. Each 20 µL PCR reaction mixture consisted of genomic DNA (20 ng), 10 µL OneTaq® Quick-Load 2X Master Mix (NEB, USA), 1 µL forward primer (10 uM), and 1 µL reverse primer (10 uM). All the procedures of gel electrophoresis, PCR-re-amplification, PCR cleanup and quantitation followed exactly the same as described previously.

RESULTS

A fragment of approximately 1.4 kb was amplified using 16S rDNA primers Fig. 1. Sequencing data was merged and consensus sequences were subjected to NCBI-BLAST which gave first best hits with *Acinetobacter* spp. (MW559450; 100% coverage and 99.93% identity) and best hits with the species *Acinetobacter calcoaceticus* (MG011543; 99% coverage and 99.93% identity; Fig. 2). Phylogenetic analysis was done using 16S rDNA nucleotide sequences of 11 different *Acinetobacter* species, which were retrieved from the Gen-Bank database. Multiple alignments of the sequences were performed using MUSCLE. The phylogenetic tree

was subsequently analyzed and displayed by Neighbor joining MEGA software. Bootstrap values were calculated from 1000 replications for statistical analysis. Based on the phylogenetic clustering the 16S rDNA sequences of FO1-S19 were clustered with other *Acinetobacter species* and claded with *Acinetobacter calcoaceticus* Fig. 3. The constructed Phylogenetic tree (Fig 3.) showed high similarity with the *Acinetobacter calcoaceticus,* that is isolated from environmental sample (water; Fig. 3).
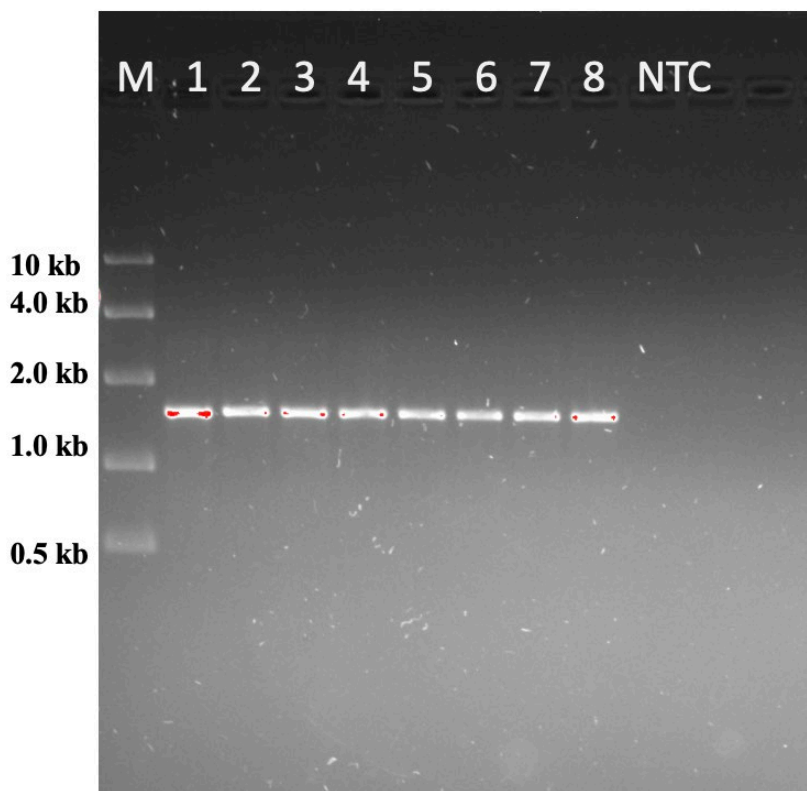

Figure 1. 16S rDNA primers were used for PCR from diluted gDNA samples (lane 1-8), M: FatsRuler DNA Ladder High Range (10 – 0.5 kb), NTC: No template control.

```
GTACCGCCCTCTTTGCAGTTAGGCTAGCTACTTCTGGTGCAACAAACTCCCATGGTGTGACGGG
CGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATTCTGATCCGCGATTACTAGCGATTC
CGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGCAT
CCTATCGCTAGGTAGCAACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAG
GGCCATGATGACTTGACGTCGTCCCCGCCTTCCTCCAGTTTGTCACTGGCAGTATCCTTAAAGT
TCCCATCCGAAATGCTGGCAAGTAAGGAAAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
```

```
CTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTATCTAGATTCCCGAAGGCACCAATCC
ATCTCTGGAAAGTTTCTAGTATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAAC
CACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAGTCTTGCGACCGTAC
TCCCCAGGCGGTCTACTTATCGCGTTAGCTGCGCCACTAAAGCCTCAAAGGCCCCAACGGCTAG
TAGACATCGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCATGCTTTCGTA
CCTCAGCGTCAGTATTAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTACGC
ATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCTTCCCAGTATCGAATG
CAATTCCCAAGTTAAGCTCGGGGATTTCACATCCGACTTAAAAAGCCGCCTACGCACGCTTTAC
GCCCAGTAA
```

Figure 2. Phylogenetic relationship of FO1-S19 based on 16S rDNA comparisons.
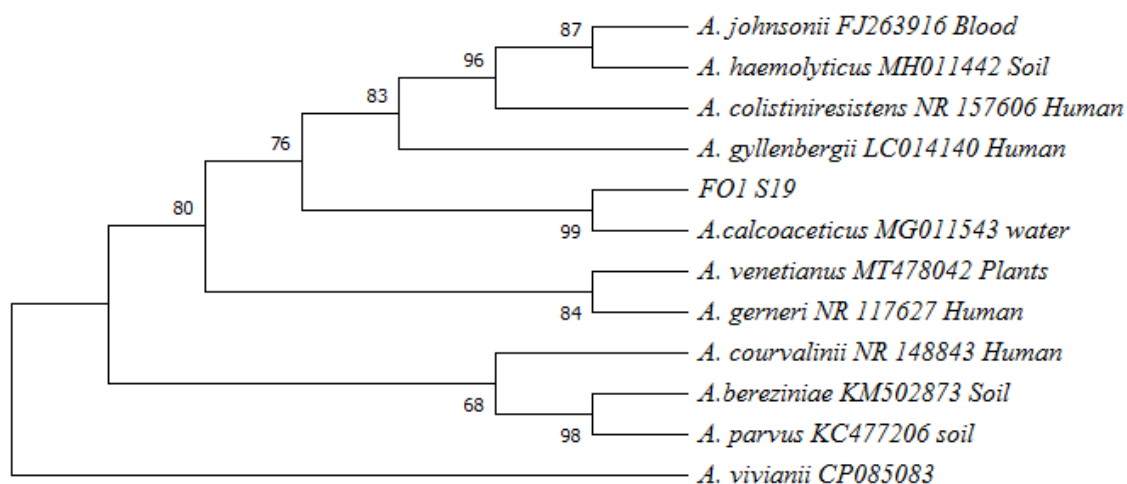

Figure 3. 16S rRNA based phylogenetic tree of FO1-S19

The genomic information of FO1-S19 is presented in Table 1. In brief, the reads were assembled using an a5-MiSeq assembler that produced sequences of 3.92 Mb in 201 contigs with an average coverage of 78 fold and 42.2% of GC content. The total number of genes in the draft genome identified were 3660, which included 68 RNA genes. Signal peptide sequences (associated with secreted proteins; Bendtsen et. al., 2004) were found in 564 gene products and 868 gene products encoded transmembrane motifs (Krogh et. al., 2001). Table 2 summarizes the predicted resistance genes detected in FO1-S19. The gene blaOXA-295 encoding for β-

lactamase and two aminoglycoside modification resistance genes (*aac6-Ir and ant(3")-IIb*) were detected. Furthermore, genes encoding for efflux pump membrane transporter *abeB, adeF,* were detected (Ju et. al., 2021). A gene encoding for an efflux pump regulator, *mexT,* was also identified and is associated with antibiotic resistance (Köhler et. al., 1999). Figure 4. shows a visualization of the genome made using GCview and laid out into a circular map. It includes the open reading frames (ORF's), GC skew, and GC percentage. We could not detect any virulence genes and plasmids in this isolate.

Table 1: Nucleotide content and gene count levels of the chromosome.

Attribute	Value	% of total
Genome size (bp)	3,920,189	100
DNA G+C content (bp)	1,618,258	41.28
Coverage	78	-
Total genes	3,660	100
RNA genes	68	1.86
Protein-coding genes	3592	98.14
Genes with function prediction	2,074	56.70
Genes with peptide signals	564	15.41
Genes with transmembrane helices	868	23.72

Table 2: Resistance gene detected in FO1-S19.

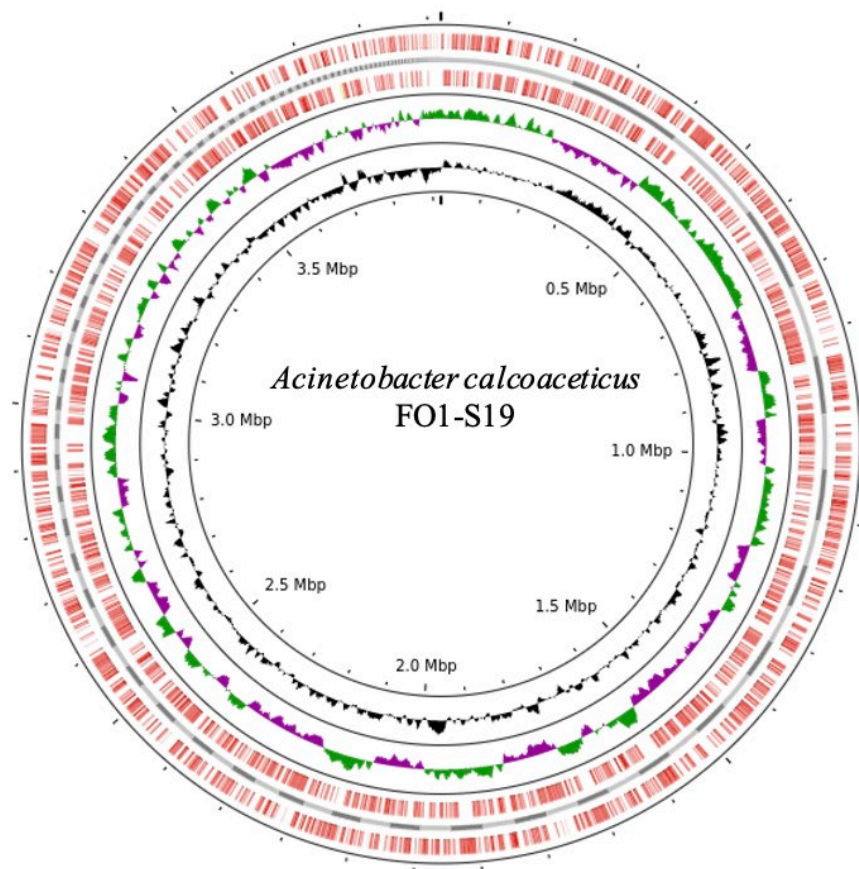| Gene	Identity (%)	comments
aac6-Ir	100	Aminoglycoside N-acetyltransferases
ant(3")-IIb	100	Aminoglycoside O-nucleotidyltransferases
blaOXA-295	100	Class D betalactamases
adeB	99.84	Drug and biocide RND efflux pumps
adeF	99.21	Drug and biocide RND efflux pumps
mexT	100	Drug and biocide RND efflux pumps

Figure 4. Genome map of the *Acinetobacter* spp. FO1-S19. From outside to the center: Genes on the forward strand (clockwise red lines), genes on the reverse strand (red lines in the penultimate circle), GC skew (pink and green), and GC content (Black).

Of the 38 additonal isolate DNA samples investigatedm only 15 produced the desired 16S rDNA amplicon, that was confirmed by agarose gel electrophoresis (1.2% agarose, 80 V). The amplified products were re-amplified and checked again on 1.2% agarose gel electrophoresis. The re-amplified PCR product were subjected to PCR cleanup using the PCR cleanup kit (NEB, USA) as per manufacturer's instructions. These samples are currently in the process of being sequenced. T10G-C3, T10G-C4, T10G-C2-2, T20G-C1, T20G-C2, T2G-C1, T23G-C1, MOID-3, FA1081-C2, H21G-C1, H21G-C3, H7F-C1, H7F-C2, H7F-C3 were all the isolates whose DNA was successfully extracted and amplified. The details of these isolates were given in Table 3.

Table 3. List of 15 microbe isolates and their source of isolation from *N. americanus*

Isolate ID	Description
T10G - C3	Female anal 1250 Colony 3
T10G - C4	Female anal 1250 Colony 4
T10G - C2	Female anal 1250 Colony 2
T20G - C1	Male anal 1082 Colony 1
T20G - C2	Male anal 1082 Colony 2
T2G - C1	Female anal 1081 Colony 1
T23G - C1	Male oral 1082 Colony 1
MOID-3	Male oral ID Colony 3
FA1081-C2	Female anal 1081 Colony 2
H21G - C1	Male anal 1082 Colony 1
H21G - C3	Male anal 1082 Colony 3
H7F - C1	Female oral 1081 Colony 1
H7F - C2	Female oral 1081 Colony 2
H7F - C3	Female oral 1081 Colony 3

DISCUSSION

The BLAST analysis of 16s rDNA sequence best matched with *A.calcoaceticus* (MG011543) and FO1-S19 closely clustered *A. calcoaceticus*. To confirm this finding, a *rpoB* based phylogenetic interpretation and average nucleotide identity (ANI) matrix could be helpful.

A. calcoaceticus is a Gram-negative bacterium that is very common in the environment. It can also be found as a commensal organism in humans. *A. calcoaciticus* is carried by up to 25% of individuals sampled (Retailliau et. al., 1979) and this species can cause disease (Glew et. al., 1977). *A. calcoaciticus* has been attributed to causing infections such as septicemia, endocarditis, brain abscess, pneumonia, tracheobronchitis, empyema, urinary tract infections, intraabdominal abscess, intrapelvic abscess, pyarthrosis, osteomyelitis, cellulitis, skin abscess, wound infection, conjunctivitis, and phlebitis (Glew et. al., 1977). *A. calcoaciticus* has also been isolated from some insects, including *Ocymyrmex velox* as a cellulase producer (Shil et. al.,

2014), *Oberea linearis* (Bahar et. al., 2007), *Lymantria dispar* (gypsy moth) (Demir et. al., 2012), *Comadia redtenbacheri* (Hernández-Flores et. al., 2015),„ *nematode Steinernema spp.* (Fu et. al., 2019), and possibly *Leptinotarsa decemlineat* (Muratoglu et. al., 2011). Since this organism has been reported to be found in other insects, it seemed appropriate we isolated it from *N. americanus*. More work is required to determine where in the beetle's anatomy that *A. calcoaciticus* is located.

As we suspected, genes encoding for antibiotic resistance were found in the genome of FO1-S19 isolate. *aac6-Ir*, *ant(3")-IIb*, *bla*OXA-295, and *mexT* were found with 100% identity where as *abeB* and *adeF* were found with 99.84% and 99.21% respectively. *Aac(6')* (Doi et. al., 2004), *ant(3")-IIb* (Zhang et. al., 2017), *blaOXA-23* (Corrêa et. al., 2012), *mexT, adeB,* and *adeF*, have all previously been detected in *Acinetobacter* spp (Thadtapond et. al., 2021). These genes contribute to the multidrug resistance phenotype characteristic of *A. calcoaciticus-baumannii* complexes (Thadtapond et. al., 2021). Its efflux pump proteins are especially of concern for antibiotic treatment because they can target a variety of drugs and make them inefficient against the bacteria (Thadtapond et. al., 2021). No known plasmid was discovered in FO1-S19 , even though Gram-negative pathogens often carry plasmids that harbor antibiotic resistance genes (Li et. al., 2019).

Overall, our study confirmed initial results that FO1-S19 is indeed an *Acinetobacter* spp. It also corroborated the presence of antibiotic resistance encoding genes in FO1-S19 which can be typical of *Acinetobacter spp.* (Van et. al., 2014, Manchanda et. al., 2010).

Complete genome alignments with multiple Acinetobacter spp. are required to determine if FO1-S19 is indeed a unique strain and would confirm its phylogenetic relationship. Additional

research is required to identify the 38 additional aerobic microbe isolates isolated from *N.*

americanus.

REFERENCES

Aalborg University Denmark. (n.d.). *Glutamicibacter*. Midas Field Guide. Retrieved May 4, 2022, from https://www.midasfieldguide.org/guide/fieldguide/genus/glutamicibacter

Abo-Zed A, Yassin M, Phan T. *Acinetobacter junii* as a rare pathogen of urinary tract infection. *Urol Case Rep*. 2020; 32:101209. (Abo-Zed et. al., 2020)

Andalibi, F., & Fatahi-Bafghi, M. (2017). Gordonia: Isolation and identification in clinical samples and role in biotechnology. *Folia Microbiologica*, *62*(3), 245–252. https://doi.org/10.1007/s12223-017-0491-1

Bahar, A. A., & Demirbağ, Z. (2007). Isolation of pathogenic bacteria from Oberea linearis (Coleptera: Cerambycidae). *Biologia*, *62*(1), 13–18. https://doi.org/10.2478/s11756-007-0009-4

Banerjee, S., Maiti, T. K., & Roy, R. N. (2020). Production, purification, and characterization of cellulase from Acinetobacter Junii GAC 16.2, a novel cellulolytic gut isolate of Gryllotalpa africana, and its effects on cotton fiber and sawdust. *Annals of Microbiology*, *70*(1). https://doi.org/10.1186/s13213-020-01569-6

Beharrysingh, R. (2017). Myroides bacteremia : A case report and concise review. *IDCases*, *8*, 34–36. https://doi.org/10.1016/j.idcr.2017.02.012

Bendtsen JD, Nielsen H, von Heijne G and Brunak S. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol. 2004; 340:783-795

Bernard, K. A., Pacheco, A. L., Loomer, C., Burdz, T., Wiebe, D., Huynh, C., Kaplen, B., Olson, A. B., Cnockaert, M., Eguchi, H., Kuwahara, T., Nakayama-Imaohji, H., Shiota, H., Boudewijns, M., Van Hoecke, F., & Vandamme, P. (2016). Corynebacterium lowii sp. nov. and Corynebacterium Oculi sp. nov., derived from human clinical disease and an emended description of Corynebacterium Mastitidis. *International Journal of Systematic and Evolutionary Microbiology*, *66*(8), 2803–2812. https://doi.org/10.1099/ijsem.0.001059

Carrie L. Hall, Nicholas K. Wadsworth, Daniel R. Howard, Eleanor M. Jennings, Larry D. Farrell, Timothy S. Magnuson, Rosemary J (2011). Smith, Inhibition of Microorganisms on a Carrion Breeding Resource: The Antimicrobial Peptide Activity of Burying Beetle (Coleoptera: Silphidae) Oral and Anal Secretions, *Environmental Entomology*, Volume 40, Issue 3, 1 June 2011, Pages 669–678, https://doi.org/10.1603/EN10137

Corrêa, L. L., Botelho, L. A., Barbosa, L. C., Mattos, C. S., Carballido, J. M., de Castro, C. L., Mondino, P. J., de Paula, G. R., de Mondino, S. S., & de Mendonça-Souza, C. R. (2012). Detection of blaoxa-23 in Acinetobacter spp. isolated from patients of a University Hospital. *The Brazilian Journal of Infectious Diseases*, *16*(6), 521–526. https://doi.org/10.1016/j.bjid.2012.10.003

D. Coil, G. Jospin, A.E. Darling A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data Bioinformatics, 31 (2015), pp. 587-589.

Doi, Y., Wachino, J.-ichi, Yamane, K., Shibata, N., Yagi, T., Shibayama, K., Kato, H., & Arakawa, Y. (2004). Spread of Novel Aminoglycoside Resistance Gene aac(6′)-Iad among Acinetobacter Clinical Isolates in Japan. *Antimicrobial Agents and Chemotherapy*, *48*(6), 2075–2080. https://doi.org/10.1128/aac.48.6.2075-2080.2004

Degenkolb, T., Düring, R.A., & Vilcinskas, A. (2011). Secondary metabolites released by the burying beetle Nicrophorus vespilloides: Chemical analyses and possible ecological functions. Journal of Chemical Ecology, 37(7), 724-35. doi: 10.1007/s10886-011-9978-4

Demir, I., Demirbag, Z., & Eryuzlu, E. (2012). A study on the characterization and pathogenicity of bacteria from Lymantria dispar L. (Lepidoptera: Lymantriidae). *Turkish Journey of Biology*, *36*(4). https://doi.org/10.3906/biy-1107-18

Drzewiecka, D. Significance and Roles of *Proteus* spp. Bacteria in Natural Environments. *Microb Ecol* 72, 741–758 (2016). https://doi.org/10.1007/s00248-015-0720-6

Euzéby, J.P. (1997). List of bacterial names with standing in nomenclature: a folder available on the internet. International Journal of Systematic Bacteriology, 47, 590-592. Doi: 10.1099/00207713-47-2-590

Fournier PE and Richet H. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. Clinical Infectious Diseases. 2006; 42(5):692–699.

Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., and Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl. Environ. Microbiol.* 74, 2461–2470. doi: 10.1128/AEM.02272-07.

Fu, J., & Liu, Q. (2019). Evaluation and entomopathogenicity of gut bacteria associated with dauer juveniles of Oscheius chongmingensis (Nematoda: Rhabditidae). *MicrobiologyOpen*, *8*(9). https://doi.org/10.1002/mbo3.823

Geiger, A., Fardeau, M.-L., Grebaut, P., Vatunga, G., Josénando, T., Herder, S., Cuny, G., Truc, P., & Ollivier, B. (2009). First isolation of Enterobacter, Enterococcus, and Acinetobacter spp. as inhabitants of the tsetse fly (glossina palpalis palpalis) midgut. *Infection, Genetics and Evolution*, *9*(6), 1364–1370. https://doi.org/10.1016/j.meegid.2009.09.013

Girlich, D., Bonnin, R. A., Dortet, L., & Naas, T. (2020). Genetics of acquired antibiotic resistance genes in proteus spp.. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.00256

Hamilton, A. L., Kamm, M. A., Ng, S. C., & Morrison, M. (2018). Proteus spp. as putative gastrointestinal pathogens. *Clinical Microbiology Reviews*, *31*(3). https://doi.org/10.1128/cmr.00085-17

Hernández-Flores, L., Llanderal-Cázares, C., Guzmán-Franco, A. W., & Aranda-Ocampo, S. (2015). Bacteria Present in Comadia redtenbacheri Larvae (Lepidoptera: Cossidae) . *Journal of Medical Entomology*, *52*(5), 1150–1158. https://doi.org/10.1093/jme/tjv099

Hoback, W. W., Bishop, A. A., Kroemer, J., Scalzitti, J., & Shaffer, J. J. (2004). Differences among antimicrobial properties of carrion beetle secretions reflect phylogeny and ecology. *Journal of Chemical Ecology*, *30*(4), 719–729. https://doi.org/10.1023/b:joec.0000028427.53141.41

Huang,X. and Madan,A. (1999) CAP3: a DNA sequence assembly program. Genome Res., 9, 868-877.

Jacobs, C. G. C., Steiger, S., Heckel, D. G., Wielsch, N., Vilcinskas, A., & Vogel, H. (2016). Sex, offspring and carcass determine antimicrobial peptide expression in the burying beetle. *Scientific Reports*. Retrieved from https://www.nature.com/articles/srep25409.

Joshi, M., Mande, S., & Dikshit, K.L. (1998). Hemoglobin biosynthesis in Vitreoscilla stercoraria DW: cloning, expression, and characterization of a new homolog of a bacterial globin gene. Applied and Environmental Microbiology, 64(6), 2220-2228. Doi: 10.1128/AEM.64.6.2220-2228.1998

Ju Y, Kim YJ, Chang CL, Choi GE, Hyun KY. Relationship between AdeABC Efflux Pump Genes and Carbapenem in Multidrug-resistant *Acinetobacter baumannii*. Biomedical Science Letters. 2021. 27: 59-68.

Kämpfer, P., Rosselló-Mora, R., Scholz, H. C., Welinder-Olsson, C., Falsen, E., & Busse, H.-J. (2006). Description of pseudochrobactrum gen. nov., with the two species Pseudochrobactrum Asaccharolyticum sp. nov. and pseudochrobactrum saccharolyticum sp. nov.. *International Journal of Systematic and Evolutionary Microbiology*, *56*(8), 1823–1829. https://doi.org/10.1099/ijs.0.64256-0

Khetani, S., & Parker, T. (2011). *Nicrophorus americanus (American burying beetle)*. Animal Diversity Web. https://animaldiversity.org/accounts/Nicrophorus_americanus/

Köhler T, Epp SF, Curty LK, Pechère JC. 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J Bacteriol 181:6300–6305

Krogh A, Larsson B, von Heijne G and Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001; 305:567-580.

Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (**2018**). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* **35**:1547-1549.

Li, Q., Chang, W., Zhang, H., Hu, D., & Wang, X. (2019). The role of plasmids in the multiple antibiotic resistance transfer in esbls-producing escherichia coli isolated from wastewater treatment plants. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.00633

Malhotra, J., Dua, A., Saxena, A., Sangwan, N., Mukherjee, U., Pandey, N., Rajagopal, R., Khurana, P., Khurana, J. P., & Lal, R. (2012). Genome sequence of Acinetobacter sp.. strain ha, isolated from the gut of the polyphagous insect pest helicoverpa armigera. *Journal of Bacteriology*, *194*(18), 5156–5156. https://doi.org/10.1128/jb.01194-12

Manchanda, V., Sinha, S., & Singh, N. P. (2010). Multidrug Resistant Acinetobacter. *Journal of Global Infectious Diseases*, *2*(3), 291–304. https://doi.org/10.4103/0974-777x.68538

MediaLab. (n.d.). *Polymerase chain reaction (PCR)*. LabCE. Retrieved from https://www.labce.com/spg538191_polymerase_chain_reaction_pcr.aspx#:~:text=Two%20 primers%20are%20utilized%2C%20one,DNA%20(the%20sense%20strand).

Minard, G., Tran, F. H., Raharimalala, F. N., Hellard, E., Ravelonandro, P., Mavingui, P., & Valiente Moro, C. (2012). Article Navigation Prevalence, genomic and metabolic profiles of Acinetobacter and Asaia associated with field-caught Aedes albopictus from Madagascar. *FEMS Microbiology Ecology*, *83*(1), 63–73. https://doi.org/10.1111/j.1574-6941.2012.01455.x

Mohd. Rani F, A. Rahman NI, Ismail S, Alattraqchi AG, Cleary DW, Clarke SC and Yeo CC (2017) *Acinetobacter spp*. Infections in Malaysia: A Review of Antimicrobial Resistance Trends, Mechanisms and Epidemiology. Front. Microbiol. 2017; 8:2479.

Muratoglu, H., Demirbag, Z., & Sezen, K. (2011). The first investigation of the diversity of bacteria associated with leptinotarsa decemlineata (Coleoptera: Chrysomelidae). *Biologia*, *66*(2), 288–293. https://doi.org/10.2478/s11756-011-0021-6

O'Hara, C. M., Brenner, F. W., and Miller, J. M. (2000). Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clin. Microbiol. Rev.* 13, 534–546.

Pratt, C. J. (2018). *Bacterial communities in carrion and burying beetle (Silphidae) secretions* (thesis). (W. Hoback, J. E. Gustafson, L. M. Ma, & F. M. Corona, Eds.)*ShareOK*. Retrieved from https://hdl.handle.net/11244/330942.

Pringsheim, E.G. (1949). The relationship between Bacteria and Myxophyceae. Bacteriological Reviews, 13(2), 47-98

Puri, A., Bajaj, A., Singh, Y., & Lal, R. (2022). Harnessing taxonomically diverse and metabolically versatile genus Paracoccus for bioplastic synthesis and xenobiotic biodegradation. *Journal of Applied Microbiology*. https://doi.org/10.1111/jam.15530

Rahal, J. J., & Urban, C. (2000). Acinetobacter. *Seminars in Respiratory and Critical Care Medicine*, *21*(04), 341–348. https://doi.org/10.1055/s-2000-9858

Rana, R.L., Hoback, W.W., Rahim, N.A.A., Bedick, J., & Stanley, D.W. (1997). Pre-oral digestion: A phospholipase A2 associated with oral secretions in adult burying beetles, Nicrophorus marginatus. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 118(2), 375-380. doi: 10.1016/S0305- 0491(97)00105-3.

Saitou N. and Nei M. (**1987**). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**:406-425. (Saitou et. al., 1987) Felsenstein J. (**1985**). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-791.

Suau, A., Bonnet, R., Sutren, M., Godon, J. J., Gibson, G. R., Collins, M. D., et al. (1999). Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl. Environ. Microbiol.* 65, 4799–4807.

Seemann T. Prokka: rapid prokaryotic genome annotation Bioinformatics 2014 Jul 15;30(14):2068-9.

Shil, R. K., Mojumder, S., Sadida, F. F., Uddin, M., & Sikdar, D. (2014). Isolation and identification of cellulolytic bacteria from the gut of three phytophagus insect species. *Brazilian Archives of Biology and Technology*, *57*(6), 927–932. https://doi.org/10.1590/s1516-8913201402620

S.K. Gupta, B.R. Padmanabhan, S.M. Diene, R. Lopez-Rojas, M. Kempf, L. Landraud, et al. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes Antimicrob Agents Chemother, 58 (2014), pp. 212-220.

Stothard P, Wishart DS (2005) Circular genome visualization and exploration using CGView. Bioinformatics 21:537-539.

Tamura K., Nei M., and Kumar S. (**2004**). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (*USA*) **101**:11030-11035.

Thadtapong, N., Chaturongakul, S., Soodvilai, S., & Dubbs, P. (2021). Colistin and carbapenem-resistant Acinetobacter baumannii ACI46 in Thailand: Genome analysis and antibiotic resistance profiling. *Antibiotics*, *10*(9), 1054. https://doi.org/10.3390/antibiotics10091054

Tripathi, P. C., Agrawal, G. N., & Gajbhiye, S. R. (2014). Clinical and antimicrobial profile of Acinetobacter spp.: An emerging nosocomial superbug. *Advanced Biomedical Research*, *3*(1), 13. https://doi.org/10.4103/2277-9175.124642

Van, T. D., Dinh, Q.-D., Vu, P. D., Nguyen, T. V., Pham, C. V., Dao, T. T., Phung, C. D., Hoang, H. T., Tang, N. T., Do, N. T., Nguyen, K. V., & Wertheim, H. (2014). Antibiotic susceptibility and molecular epidemiology of Acinetobacter calcoaceticus–baumannii complex strains isolated from a referral hospital in northern Vietnam. *Journal of Global Antimicrobial Resistance*, *2*(4), 318–321. https://doi.org/10.1016/j.jgar.2014.05.

Vogel, H., Shukla, S.P., Engl, T., Weiss, B., Fischer, R., Steiger, S., Heckel, D.G., Kaltenpoth, M., & Vilcinskas, A. (2017). The digestive and defensive basis of carcass utilization by the burying beetle and its microbiota. Nature Communications, 8, 15186. Doi: 10.1038/ncomms15186

Wang, Z., Xin, X., Shi, X., & Zhang, Y. (2020). A polystyrene-degrading acinetobacter bacterium isolated from the larvae of Tribolium Castaneum. *Science of The Total Environment*, *726*, 138564. https://doi.org/10.1016/j.scitotenv.2020.138564

Zhang, G., Leclercq, S. O., Tian, J., Wang, C., Yahara, K., Ai, G., Liu, S., & Feng, J. (2017). A new subclass of intrinsic aminoglycoside nucleotidyltransferases, ANT(3")-II, is horizontally transferred among Acinetobacter spp. by homologous recombination. *PLOS Genetics*, *13*(2). https://doi.org/10.1371/journal.pgen.1006602