Insight into the resistome and quorum sensing system of a divergent *Acinetobacter pittii* isolate from an untouched site of the Lechuguilla Cave

Han Ming Gan[1,2,3*], Peter Wengert[4] ,Trevor Penix[4], Hazel A. Barton[5], André O. Hudson[4] and Michael A. Savka[4]

[1] Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong 3220 ,Victoria, Australia

[2] Deakin Genomics Centre, Deakin University, Geelong 3220 ,Victoria, Australia

[3] School of Science, Monash University Malaysia, Bandar Sunway, 47500 Petaling Jaya, Selangor, Malaysia

[4] Thomas H. Gosnell School of Life Sciences, Rochester Institute of Technology, Rochester, NY, USA

[5] Department of Biology, University of Akron, Akron, Ohio, USA

*Corresponding author

Name: Han Ming Gan

Email: han.gan@deakin.edu.au

Key words

Acinetobacter, quorum sensing, antibiotic resistance

Abstract

Acinetobacter are Gram-negative bacteria belonging to the sub-phyla Gammaproteobacteria, commonly associated with soils, animal feeds and water. Some members of the *Acinetobacter* have been implicated in hospital-acquired infections, with broad-spectrum antibiotic resistance. Here we report the whole genome sequence of LC510, an *Acinetobacter* species isolated from deep within a pristine location of the Lechuguilla Cave. Pairwise nucleotide comparison to three type strains within *Acinetobacter* assigned LC510 as an *Acinetobacter pittii* isolate. Scanning of the LC510 genome identified two genes coding for β-lactamase resistance, despite the fact that LC510 was isolated from a portion of the cave not previously visited by humans and protected from anthropogenic input. The ability to produce acyl-homoserine lactone (AHL) signal in culture medium, an observation that is consistent with the identification of the *luxI* and *luxR* homologs in its genome, suggests that cell-to-cell communication remains important in an isolated cave ecosystem.

Introduction

The identification and functionality of antibiotic-resistant and quorum sensing genes from bacteria isolated from pristine environments (areas previously not visited by humans) have raised questions about their origins and natural functions in the environment [1, 2]. For example, several bacterial strains isolated from an extremely isolated, hyper-oligotrophic underground ecosystem, Lechuguilla Cave, were shown to harbor antibiotic-resistant genes, including nine previously unrecognized mechanisms of antibiotic resistance [1]. Previously we sequenced and annotated the whole genome sequences of four Alphaproteobacteria and one Betaproteobacterium from Lechuguilla Cave (LC), leading to the taxonomic revision of some LC strains and identification of new *luxI* genes [3]. Based on the initial 16S rRNA gene-based identification of LC strains [4], LC510 stood out due to its affiliation to the genus *Acinetobacter.* LC510 was isolated from a site deep within the Capitan Formation proximal to the region named "Deep Secrets" at a depth below the surface of approximately 400 m. Although most members of *Acinetobacter* are found in soils, waters and occasionally animal feeds, some *Acinetobacter* species are known to infect humans with broad-spectrum antibiotic resistance and such environmental isolates may serve as a reservoir for additional resistance determinants [5, 6]. In this study, we characterized LC510 using whole genome sequencing, biochemical assays, and bioinformatic tools, providing insights into its taxonomic affiliation, resistome and quorum sensing potential.

Materials and Methods:

DNA extraction and whole genome sequencing

The isolation, antibiotic characterization and 16S rRNA gene-based identification of strain LC510 have been described previously [4]. An overnight culture of LC510 in ½ strength tryptic soy broth (TSB) overnight was pelleted by centrifugation at 10,000 rpm for 10 minutes. Genomic DNA (gDNA) extraction was performed on the pelleted cells using the Qiagen Minamp kit according to the manufacturer's instructions. The purified gDNA was quantified using the Qubit BR Assay (Invitrogen) and normalized to 0.2 ng/uL for Nextera XT library preparation (Illumina, San Diego, CA). The constructed library was sequenced on an Illumina MiSeq (2 x 151 bp run configuration) located at the Monash University Malaysia Genomics Facility that routinely sequences metazoan mitogenomes [7-9] and occasionally viral and microbial genomes [10, 11] with no prior history of processing any member from the genus *Acinetobacter,* or more broadly the family Moraxellaceae.

De novo assembly and genome-based species classification

Raw paired-end reads were adapter-trimmed using Trimmomatics v0.36 [12] followed by *de novo* assembly using Unicycler v0.4.7 (default setting with minimum contig length set to 500 bp) [13]. We then used Jspecies v1.2.1 [14] to calculate the pairwise average nucleotide identity (ANI) of LC510 against the type strain genomes of *Acinebacter pittii*, *Acinetobacter lactucae,* and *Acinetobacter coaceticus*.

Genome annotation and detection of antibiotic resistance genes

Genome annotation used Prokka v1.13 [15] and the predicted protein-coding genes were used as the input for Abricate v0.8.7 (https://github.com/tseemann/abricate) to search for antibiotic resistance genes against the ResFinder database (minimum query length coverage and nucleotide identity of 90%) [16]. The alignment of bla_{OXA} proteins used MUSCLE followed by maximum likelihood tree construction with FastTree2 [17].

Acyl-homoserine lactone bioassay

LC510 was grown in liquid ½ strength TSB medium at 150 rpm overnight at 30°C. Visualization and detection of acyl homoserine lactone using thin layer chromatography and TraR-dependent *lacZ Agrobacterium tumefaciens* reporter strain was performed as previously described [18].

***In-silico* analysis of the homoserine lactone synthase gene**

Proteins exhibiting significant alignment to the PF00765 (Autoind_synth) Pfam profile were identified using HMMsearch v3.1b1 with an E-value cutoff of 1E-5 [19, 20]. The gene organization of contigs containing the *luxI* and *luxR* homolog was visualized using Easyfig (blastN setting) [21].

Results and Discussion

Genome statistics and taxonomic assignment

The assembled LC510 genome was contained in 115 contigs (N_{50} length of 66.9 Kb) with a total length and GC content of 3.8 Mb and 38.63%, respectively. Based on 16S rRNA gene identification, strain LC510 was previously assigned to the species *Acinetobacter calcoaceticus* [4]. However, it only exhibited a pairwise ANI of 90% to *Acinetobacter calcoaceticus* DSM30006^T, a value that is far below the established threshold required for species assignment [14]. Expanding the ANI calculation to other closely related species of *A. calcoaceticus* showed that strain LC510 should instead be assigned to the species *A. pittii*, given its >95% pairwise ANI to *A. pittii* DSM25618^T and *A. pittii* PHEA-2 (Figure 1).

Identification of beta-lactamase producing gene

LC510 was previously shown to exhibit resistance against ampicillin and cephalexin [4]. Scanning of its genome identified two genes coding for beta-lactamase namely $bla_{OXA-213}$-like (locus tag: YA64_005895) and bla_{ADC} (locus tag: YA64_000855). The $bla_{oxa-213-like}$ gene is commonly found among members of *Acinetobacter calcoaceticus* and *Acinetobacter pittii* (Figure 2) with demonstrated resistance to ampicillin through heterologous expression in *Escherichia coli* host [22]. A conserved penicillin-binding domain was identified in the $bla_{oxa-213-like}$ protein of LC510 which provides additional support to its role in conferring resistance to ampicillin. The resistance of LC510 to cephalexin is likely explained by the presence and expression of a bla_{ADC} gene encoding for AmpC beta-lactamase [23]. Cloning and regulated expression of these two *bla* genes will be instructive to verify their *in-silico* predicted role in hydrolyzing beta-lactam drugs [23]. The presence of bla_{ADC} and $bla_{OXA-213-like}$ genes in cave isolate LC510 that has no prior history of anthropogenic exposure supports previous work claiming that these genes contribute to the intrinsic antibiotic resistance in *A. pittii* [5, 22]. Interestingly, despite harboring a variant ($bla_{oxa-421}$) of the $bla_{OXA-213}$-like gene, the carbapenem-resistant *A. pittii* CR12-42 is susceptible to ampicillin when

supplemented with sulbactam [6, 24], indicating the susceptibility of $bla_{OXA\text{-}213\text{-}like}$ protein to a *β*-lactamase inhibitor [24].

AHL synthesis

Given the demonstrated ability of several Acinetobacter strains to produce quorum sensing signal that is implicated in the regulation of virulence factors and cell motility, we used both *in silico* and *in vivo* approaches to assess the presence of quorum sensing system in strain LC510. Under the described culturing condition, LC510 strain appears to produce a medium-length AHL signal that exhibits a migration rate between C6-OH and C8-OH (Figure 3). The *luxI* and *luxR* homologs in LC510 were localized on contigs 41 and 18, respectively that exhibit strikingly high synteny to the luxI/*luxR* gene cluster in *A. pittii* PHEA-2 (Figure 4). Such a gene organization was similarly found in *Acinetobacter baumannii* M2 [25], hinting the conservation of this gene cluster and its quorum sensing (QS)-regulated genes among members of *Acinetobacter*. Transposon disruption of the *luxI* homolog (*abaI*) in strain M2 led to a substantial reduction in motility that could be rescued with the supplementation of its cognate AHL signal in the media [25]. The presence of this gene cluster in LC510 may suggest that the role of quorum sensing in regulating the motility of LC510 in its cave environment. The construction of *luxI* mutant for LC510, using either transposon mutagenesis or homologous recombination [26, 27], followed by transcriptome sequencing [28] will be extremely useful not only for validating the role of QS in cell motility but also in discovering other genes and phenotypes that may be regulated by QS.

Data Availability

Raw paired-end sequencing reads and genome assembly have been deposited into the NCBI public database under the BioProject ID PRJNA281683 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA281683/).

Acknowledgments

PD, TP, AOH, and MAS acknowledge the College of Science and the Thomas H. Gosnell School of Life Sciences for support.

Figure Legends

Figure 1. Heatmap showing the clustering of *Acinetobacter* isolates based on their pairwise average nucleotide identity as indicated by the numerical values in the boxes.

Figure 2. Maximum likelihood tree depicting the evolutionary relationships of *bla* proteins found in *Acinetobacter* species. Boxed clades indicate the OXA-231-like proteins from *Acinetobacter pittii* and the tree were rooted with OXA-143-like and OXA-235-like proteins as the outgroup [29]. Tip labels were formatted as “GenBank Accession Number”~ “OXA variant” _ “[strain species and ID]”. Branch lengths indicate the number of substitutions per site.

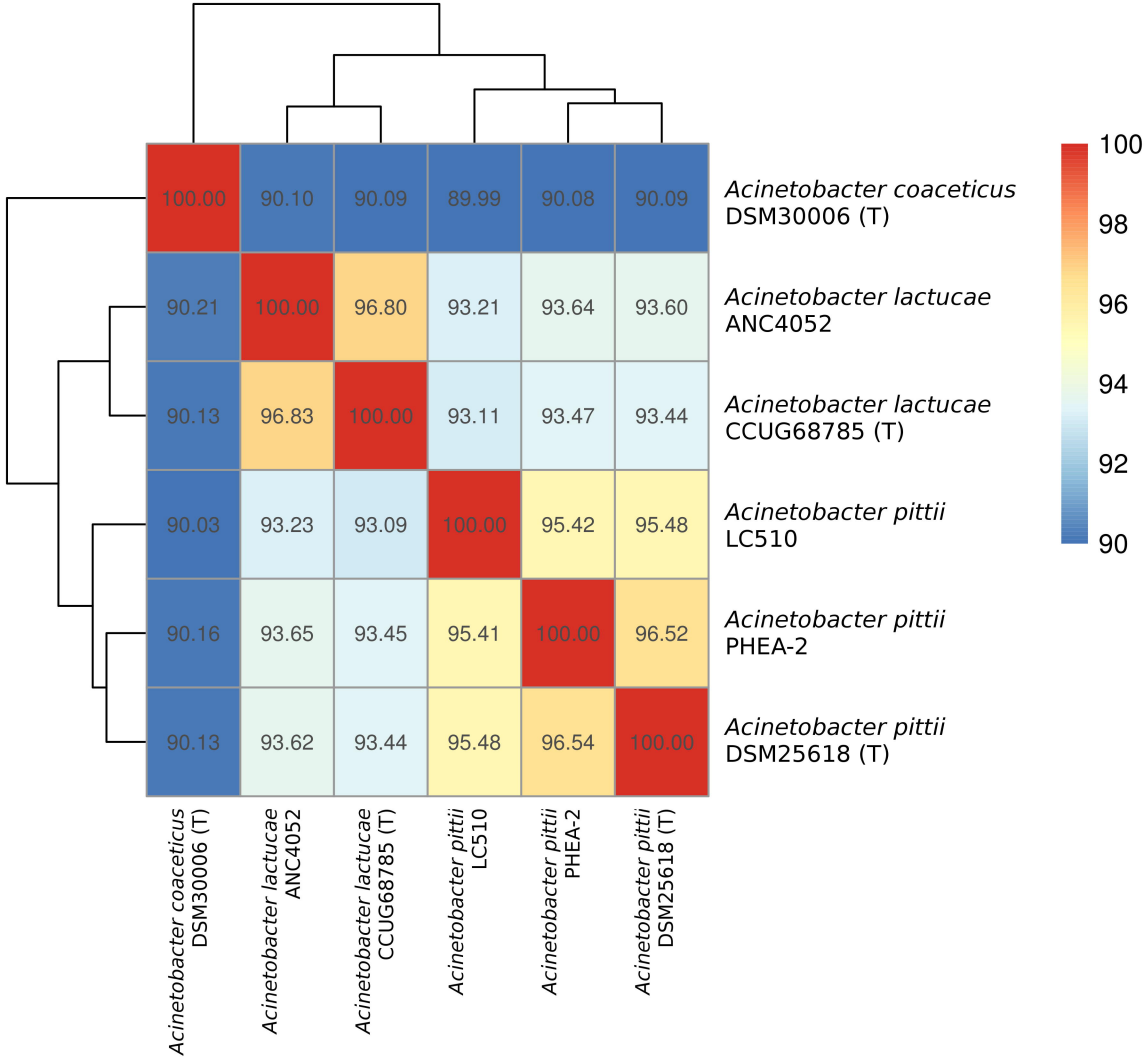
Figure 3. Thin Layer Chromatography of AHL signal extract of LC510 and known AHL standards separated using 70:30 Methanol:Water and overlaid with *Agrobacterium tumefaciens* biosensor in the presence of β-galactosidase substrate, X-Gal [18]. 2O µl of 20x ethyl acetate culture extract equivalent to 400 mL of the overnight LC510 culture was spotted on Lane 4. C6, N-Hexanoyl-L-homoserine lactone; C8, N-octanoyl-L-Homoserine lactone; C6-oxo, N-β-oxo-Hexanoyl-L-homoserine lactone; C8-oxo, N-β-oxo-octanoyl-L-Homoserine lactone; C6-OH, N-(3-hydroxy-hexanoyl)-L-homoserine lactone; C8, N-(3-hydroxy-octanoyl)-L-Homoserine lactone.

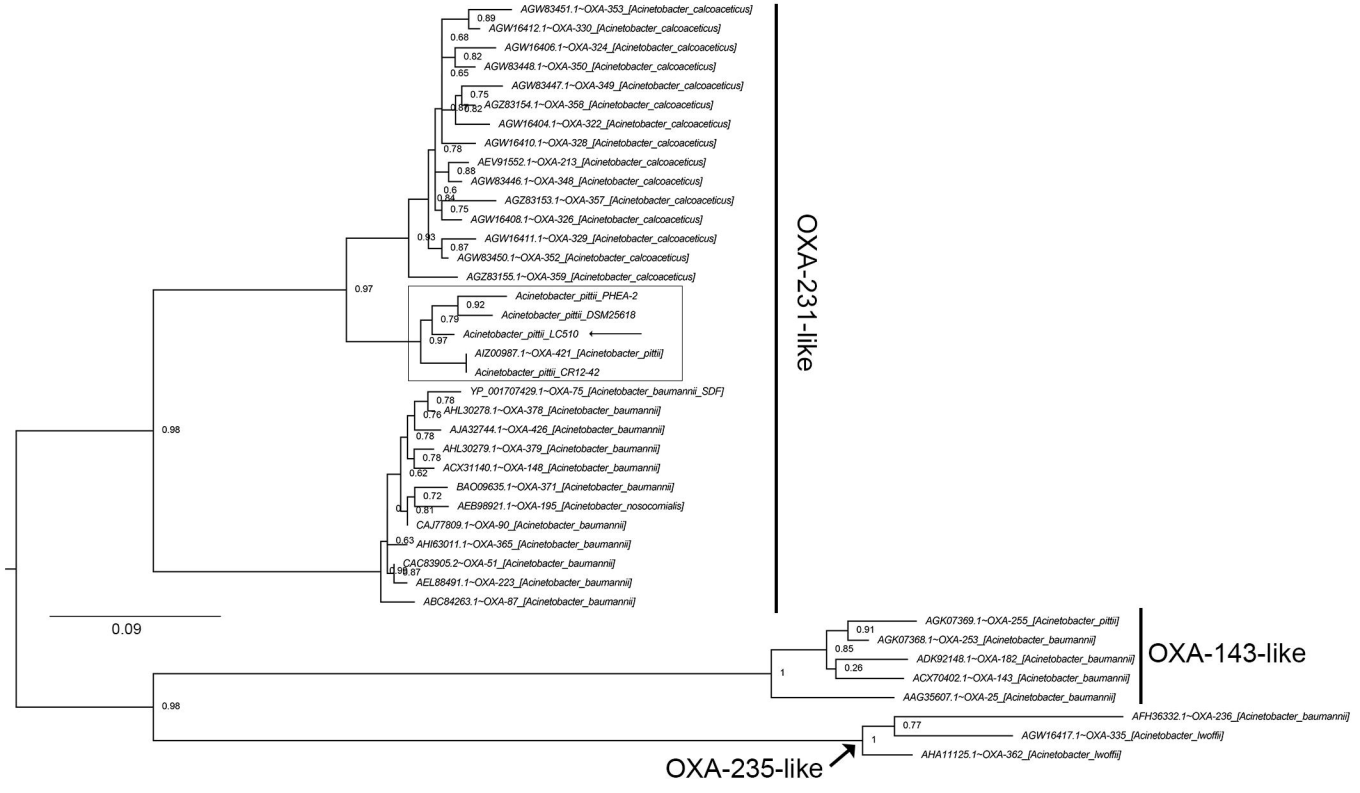
Figure 4. Linear genome comparison of the LC510 contigs containing the *luxR* and *luxI* homologs with closely related *Acinetobacter pittii* PHEA-2 strain. The directions of the arrows indicate transcription orientation. Note: LC510 contigs 41 and 18 contain *luxI* and *luxR* homolog, respectively.

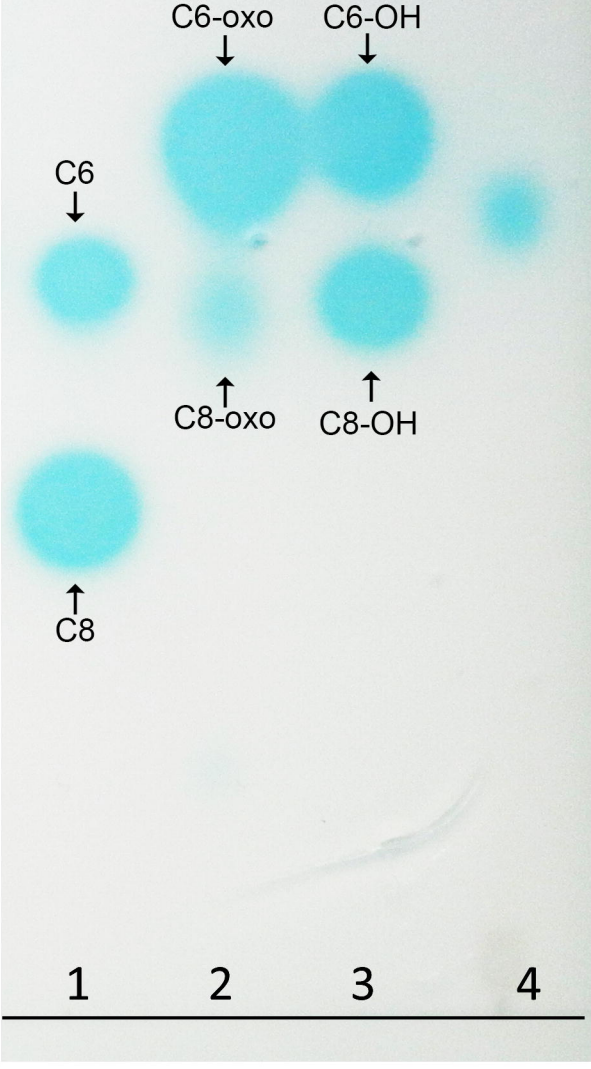
References

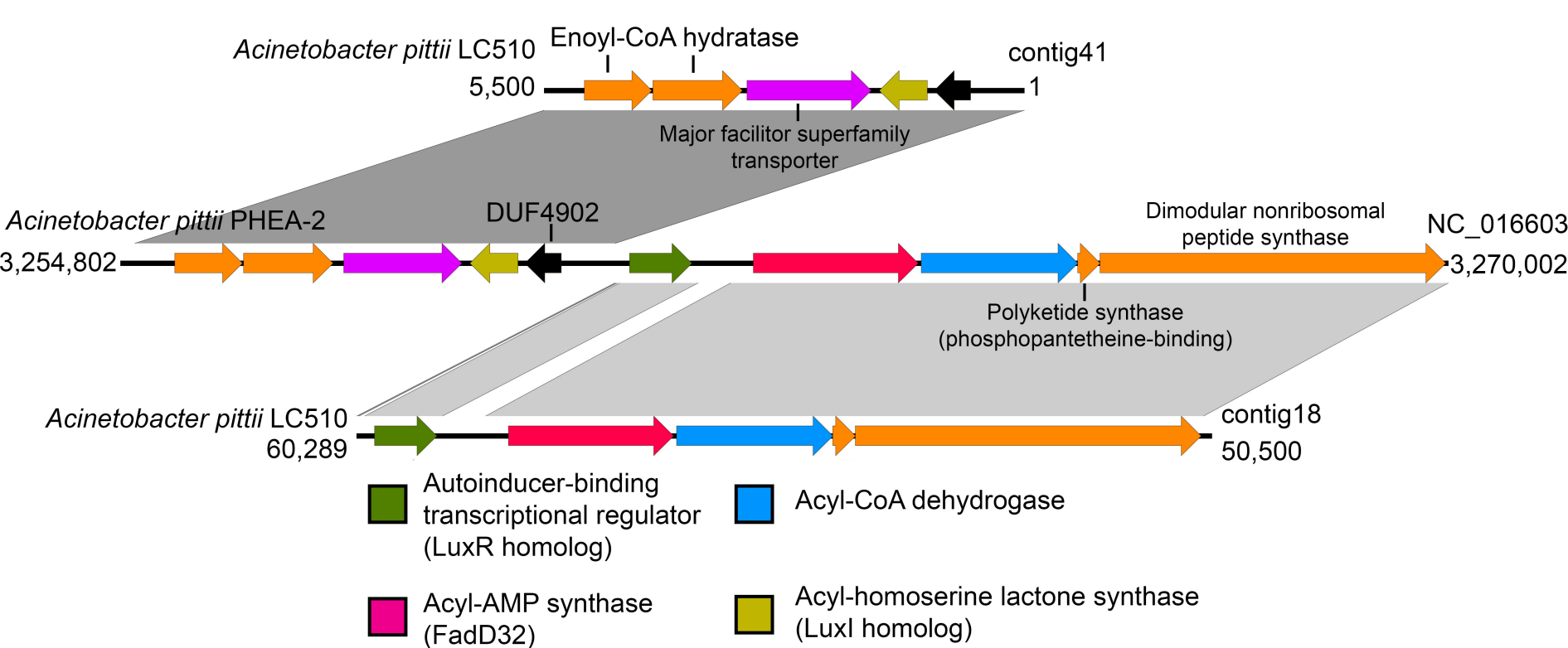
1. Pawlowski AC, Wang W, Koteva K, Barton HA, McArthur AG, Wright GD. A diverse intrinsic antibiotic resistome from a cave bacterium. Nature communications. 2016; 7: 13803.
2. Peterson E, Kaur P. Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. Frontiers in Microbiology. 2018; 9.
3. Gan HY, Gan HM, Tarasco AM, Busairi NI, Barton HA, Hudson AO, et al. Whole-Genome Sequences of Five Oligotrophic Bacteria Isolated from Deep within Lechuguilla Cave, New Mexico. Genome announcements. 2014; 2: e01133-14.
4. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, Johnston MD, et al. Antibiotic Resistance Is Prevalent in an Isolated Cave Microbiome. PLOS ONE. 2012; 7: e34953.
5. Cho G-S, Li B, Rostalsky A, Fiedler G, Rösch N, Igbinosa E, et al. Diversity and Antibiotic Susceptibility of *Acinetobacter* Strains From Milk Powder Produced in Germany. Frontiers in microbiology. 2018; 9: 536-.
6. Gonzalez C, Garcia A, Urrea R, del Solar E, Bello H, Zemelman R. A combination of ampicillin and sulbactam: effect on aerobic and anaerobic gram-negative bacteria. Revista medica de Chile. 1990; 118: 548-54.
7. Grandjean F, Tan MH, Gan HM, Lee YP, Kawai T, Distefano RJ, et al. Rapid recovery of nuclear and mitochondrial genes by genome skimming from Northern Hemisphere freshwater crayfish. Zoologica Scripta. 2017; 46: 718-28.
8. Tan MH, Gan HM, Schultz MB, Austin CM. MitoPhAST, a new automated mitogenomic phylogeny tool in the post-genomic era with a case study of 89 decapod mitogenomes including eight new freshwater crayfish mitogenomes. Molecular Phylogenetics and Evolution. 2015; 85: 180-8.
9. Martins RF, Fickel J, Le M, van Nguyen T, Nguyen HM, Timmins R, et al. Phylogeography of red muntjacs reveals three distinct mitochondrial lineages. BMC Evolutionary Biology. 2017; 17: 34.
10. Dhanoa A, Hassan SS, Ngim CF, Lau CF, Chan TS, Adnan NAA, et al. Impact of dengue virus (DENV) co-infection on clinical manifestations, disease severity and laboratory parameters. BMC infectious diseases. 2016; 16: 406.
11. Gan HY, Gan HM, Savka MA, Triassi AJ, Wheatley MS, Smart LB, et al. Whole-genome sequences of 13 endophytic bacteria isolated from shrub willow (*Salix*) grown in Geneva, New York. Genome Announc. 2014; 2: e00288-14.

12. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30: 2114-20.
13. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS computational biology. 2017; 13: e1005595.
14. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics. 2015; 32: 929-31.
15. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014; 30: 2068-9.
16. Kleinheinz KA, Joensen KG, Larsen MV. Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and E. coli virulence genes in bacteriophage and prophage nucleotide sequences. Bacteriophage. 2014; 4: e27943.
17. Price MN, Dehal PS, Arkin AP. FastTree 2–approximately maximum-likelihood trees for large alignments. PloS one. 2010; 5: e9490.
18. Lowe N, Gan HM, Chakravartty V, Scott R, Szegedi E, Burr TJ, et al. Quorum-sensing signal production by *Agrobacterium vitis* strains and their tumor-inducing and tartrate-catabolic plasmids. FEMS microbiology letters. 2009; 296: 102-9.
19. Johnson LS, Eddy SR, Portugaly E. Hidden Markov model speed heuristic and iterative HMM search procedure. BMC bioinformatics. 2010; 11: 431.
20. Gan HM, Gan HY, Ahmad NH, Aziz NA, Hudson AO, Savka MA. Whole genome sequencing and analysis reveal insights into the genetic structure, diversity and evolutionary relatedness of luxI and luxR homologs in bacteria belonging to the Sphingomonadaceae family. Frontiers in cellular and infection microbiology. 2015; 4: 188.
21. Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. Bioinformatics. 2011; 27: 1009-10.
22. Périchon B, Goussard S, Walewski V, Krizova L, Cerqueira G, Murphy C, et al. Identification of 50 Class D β-Lactamases and 65 *Acinetobacter*-Derived Cephalosporinases in *Acinetobacter* spp. Antimicrobial Agents and Chemotherapy. 2014; 58: 936-49.
23. Lopes B, Amyes S. Role of ISAba1 and ISAba125 in governing the expression of blaADC in clinically relevant *Acinetobacter baumannii* strains resistant to cephalosporins. Journal of medical microbiology. 2012; 61: 1103-8.
24. Kamolvit W, Derrington P, Paterson DL, Sidjabat HE. A case of IMP-4-, OXA-421-, OXA-96-, and CARB-2-producing Acinetobacter pittii sequence type 119 in Australia. Journal of clinical microbiology. 2015; 53: 727-30.
25. Clemmer KM, Bonomo RA, Rather PN. Genetic analysis of surface motility in *Acinetobacter baumannii*. Microbiology (Reading, England). 2011; 157: 2534-44.
26. Marx CJ. Development of a broad-host-range *sacB*-based vector for unmarked allelic exchange. BMC research notes. 2008; 1: 1.
27. Gan HM, Ibrahim Z, Shahir S, Yahya A. Identification of genes involved in the 4-aminobenzenesulfonate degradation pathway of Hydrogenophaga sp. PBC via transposon mutagenesis. FEMS microbiology letters. 2011; 318: 108-14.
28. Gao R, Krysciak D, Petersen K, Utpatel C, Knapp A, Schmeisser C, et al. Genome-wide RNA sequencing analysis of quorum sensing-controlled regulons in the plant-associated *Burkholderia glumae* PG1 strain. Appl Environ Microbiol. 2015; 81: 7993-8007.
29. Evans BA, Amyes SG. OXA β-lactamases. Clinical microbiology reviews. 2014; 27: 241-63.


100.00 90.10 90.09 89.99 90.08 90.09
90.21 100.00 96.80 93.21 93.64 93.60
90.13 96.83 100.00 93.11 93.47 93.44
90.03 93.23 93.09 100.00 95.42 95.48
90.16 93.65 93.45 95.41 100.00 96.52
90.13 93.62 93.44 95.48 96.54 100.00
Acinetobacter coaceticus DSM30006 (T)
Acinetobacter lactucae ANC4052
Acinetobacter lactucae CCUG68785 (T)
Acinetobacter pittii LC510
Acinetobacter pittii PHEA-2
Acinetobacter pittii DSM25618 (T)
100
98
96
94
92
90


AGW83451.1~OXA-353_[Acinetobacter_calcoaceticus]
AGW16412.1~OXA-330_[Acinetobacter_calcoaceticus]
AGW16406.1~OXA-324_[Acinetobacter_calcoaceticus]
AGW83448.1~OXA-350_[Acinetobacter_calcoaceticus]
AGW83447.1~OXA-349_[Acinetobacter_calcoaceticus]
AGZ83154.1~OXA-358_[Acinetobacter_calcoaceticus]
AGW16404.1~OXA-322_[Acinetobacter_calcoaceticus]
AGW16410.1~OXA-328_[Acinetobacter_calcoaceticus]
AEV91552.1~OXA-213_[Acinetobacter_calcoaceticus]
AGW83446.1~OXA-348_[Acinetobacter_calcoaceticus]
AGZ83153.1~OXA-357_[Acinetobacter_calcoaceticus]
AGW16408.1~OXA-326_[Acinetobacter_calcoaceticus]
AGW16411.1~OXA-329_[Acinetobacter_calcoaceticus]
AGW83450.1~OXA-352_[Acinetobacter_calcoaceticus]
AGZ83155.1~OXA-359_[Acinetobacter_calcoaceticus]
Acinetobacter_pittii_PHEA-2
Acinetobacter_pittii_DSM25618
Acinetobacter_pittii_LC510
AIZ00987.1~OXA-421_[Acinetobacter_pittii]
Acinetobacter_pittii_CR12-42
YP_001707429.1~OXA-75_[Acinetobacter_baumannii_SDF]
AHL30278.1~OXA-378_[Acinetobacter_baumannii]
AJA32744.1~OXA-426_[Acinetobacter_baumannii]
AHL30279.1~OXA-379_[Acinetobacter_baumannii]
ACX31140.1~OXA-148_[Acinetobacter_baumannii]
BAO09635.1~OXA-371_[Acinetobacter_baumannii]
AEB98921.1~OXA-195_[Acinetobacter_nosocomialis]
CAJ77809.1~OXA-90_[Acinetobacter_baumannii]
AHI63011.1~OXA-365_[Acinetobacter_baumannii]
CAC83905.2~OXA-51_[Acinetobacter_baumannii]
AEL88491.1~OXA-223_[Acinetobacter_baumannii]
ABC84263.1~OXA-87_[Acinetobacter_baumannii]
OXA-231-like
0.09
AGK07369.1~OXA-255_[Acinetobacter_pittii]
AGK07368.1~OXA-253_[Acinetobacter_baumannii]
ADK92148.1~OXA-182_[Acinetobacter_baumannii]
ACX70402.1~OXA-143_[Acinetobacter_baumannii]
AAG35607.1~OXA-25_[Acinetobacter_baumannii]
OXA-143-like
AFH36332.1~OXA-236_[Acinetobacter_baumannii]
AGW16417.1~OXA-335_[Acinetobacter_lwoffii]
AHA11125.1~OXA-362_[Acinetobacter_lwoffii]
OXA-235-like


C6-oxo
C6-OH
C6
C8-oxo
C8-OH
C8
1
2
3
4


Acinetobacter pittii LC510
Enoyl-CoA hydratase
contig41
5,500
1
Major facilitor superfamily transporter
Acinetobacter pittii PHEA-2
DUF4902
Dimodular nonribosomal peptide synthase
NC_016603
3,254,802
3,270,002
Polyketide synthase (phosphopantetheine-binding)
Acinetobacter pittii LC510
contig18
60,289
50,500
Autoinducer-binding transcriptional regulator (LuxR homolog)
Acyl-CoA dehydrogase
Acyl-AMP synthase (FadD32)
Acyl-homoserine lactone synthase (LuxI homolog)