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Genomic and transcriptomic variation in *Bordetella* spp. following induction of erythromycin resistance

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Background: The emergence of macrolide resistance in *Bordetella pertussis*, the causative agent of pertussis, due to mutations in the 23S rRNA gene has been recently recognized. However, resistance mechanisms to macrolides in *Bordetella parapertussis* and *Bordetella holmesii* remain unknown.

Objectives: This study investigated genomic changes induced by *in vitro* exposure to erythromycin in these three main pathogens responsible for pertussis-like disease.

Methods: A set of 10 clinical and reference strains of *B. pertussis*, *B. parapertussis* and *B. holmesii* was exposed to erythromycin for 15 weeks or 30 subculture passages. Antibiotic pressure was achieved by growth on the selective media with erythromycin Etest strips or impregnated discs. Genome polymorphisms and transcriptomic profiles were examined by short- and long-read sequencing of passaged isolates.

Results: *B. parapertussis* and *B. holmesii* isolates developed significant *in vitro* resistance to erythromycin (MIC >256 mg/L) within 2 to 7 weeks and at 5 to 12 weeks, respectively. *B. pertussis* remained phenotypically susceptible to the antibiotic following 15 weeks of exposure, with the MIC between 0.032 to 0.38 mg/L. Genomic analysis revealed that *B. holmesii* developed resistance due to mutations in the 23S rRNA gene. The resistance mechanism in *B. parapertussis* was hypothesized as being due to upregulation of an efflux pump mechanism.

Conclusions: These findings indicate that both *B. holmesii* and *B. parapertussis* can be more prone to induced resistance following exposure to treatment with erythromycin than *B. pertussis*. The surveillance of macrolide resistance in *Bordetella* isolates recovered from patients with pertussis, especially persistent disease, is warranted.

Introduction

The *Bordetella* genus is comprised of several species and includes the mammalian pathogens *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica*. The human pathogens, *B. pertussis* and *B. parapertussis,* are the causative agents of pertussis, a highly infectious respiratory disease associated with prolonged coughing episodes.[1] *B. pertussis* is the primary cause of pertussis, however it is estimated that *B. parapertussis* is responsible for approximately 1% of pertussis cases worldwide.[2] In recent years, the emergence of a closely related species *Bordetella holmesii*, has impacted *B. pertussis* surveillance, as both species contain the PCR target used to diagnose *B. pertussis* infections. In Australia, *B. holmesii* has a prevalence of between 0%–16.8% and this reflects its prevalence in other developed countries.[3,4]

The currently recommended treatment for pertussis infections and post-exposure prophylaxis are macrolide antibiotics. However, macrolide-resistant strains of *B. pertussis* have been reported for some years in the USA,[5] France,[6] China,[7–9] Iran[10] and Vietnam.[11] The resistance is due to a A2037G mutation in the 23S rRNA gene of *B. pertussis* in comparison with *B. pertussis* Tohama I.[5,9] The increased prevalence of these strains in recent years has raised concerns for their global expansion.[12] Given pertussis can

Table 1. Summary of *B. pertussis* strains, year of isolation, vaccine antigen alleles, MLST type and initial MIC of erythromycin

Isolate	Year	*ptxP*	*ptxA*	*prn*	*fhaB*	*fim2*	*fim3*	MLST	Initial MIC (mg/L)	Sequencing technology
CIDM-BP1	1954	1	4	1	1	1	1	1	0.032	Illumina
CIDM-BP2	2011	3	1	2	2	1	3	2	0.032	Illumina & Nanopore
CIDM-BP3	2015	1	1	1	2	3	2	2	0.016	Illumina & Nanopore
CIDM-BP4	2015	3	1	2	2	1	1	2	0.064	Illumina

Table 2. *B. parapertussis* and *B. holmesii* isolates used in the study with their year of isolation and initial MIC of erythromycin

Strains	Year of isolation	Initial MIC (mg/L)	Sequencing technology
Bordetella parapertussis			
CIDM-BPP1	Unknown	0.125–0.19	Illumina
CIDM-BPP2	1993	0.125–0.19	Illumina & Nanopore
CIDM-BPP3	Unknown	1	Illumina
Bordetella holmesii			
CIDM-BH1	2000	0.125–0.19	Illumina
CIDM-BH2	2014	0.047–0.064	Illumina
CIDM-BH3	2016	0.25	Illumina & Nanopore

also be caused by other *Bordetella* species, namely, *B. parapertussis* and *B. holmesii*, the ability to recognize and monitor macrolide resistance in clinical strains of all *Bordetella* spp. becomes crucial.

This study examined the comparative ability of several strains of *B. pertussis* and other significant *Bordetella* spp. (*B. parapertussis* and *B. holmesii*) to develop induced phenotypic resistance following exposure to erythromycin *in vitro*. Further strains were sequenced and the genomes interrogated for any potential variation that may indicate erythromycin resistance.

Materials and Methods

Strain selection and culture conditions

A set of clinical and reference isolates was selected for antibiotic resistance induction—four *B. pertussis* strains (CIDM-BP1, CIDM-BP2, CIDM-BP3 and CIDM-BP4), three *B. parapertussis* strains (CIDM-BPP1, CIDM-BPP2 and CIDM-BPP3) and three *B. holmesii* strains (CIDM-BH1, CIDM-BH2 and CIDM-BH3). *B. pertussis* strains were chosen based on MLST,[13] SNP and vaccine antigen types[14] in order to represent currently co-circulating genotypes of the pathogen (Table 1). *B. parapertussis* and *B. holmesii* were chosen based on availability in the culture collection (Table 2).

B. pertussis and *B. parapertussis* isolates were cultured on Charcoal Blood Agar without cephalexin (CBA) and *B. holmesii* on Horse Blood Agar (HBA) (ThermoFisher Scientific, USA). The cultures were incubated at 37°C for 3–4 days aerobically.

Induction of in vitro resistance

The strains were subcultured every 3–4 days. Briefly, a suspension equivalent to a 0.5 McFarland (MF) standard was made from bacterial colonies at the edge of the inhibition zone. A fresh (either HBA or CBA) plate was inoculated with the suspension and either erythromycin Etests (BioMérieux, France) or erythromycin-impregnated discs (BioMérieux, France) were used to provide antibiotic pressure (Figure S1, available as Supplementary data at *JAC* Online). Resistance to erythromycin was defined by an MIC >0.125 mg/L[15,16] and consistently recorded for two or more passages. A total of 30 passages were performed, however, once antibiotic resistance was observed, the strain was plated on CBA or HBA without antibiotics and DNA was extracted within 48 h after inoculation. In parallel, the initial isolate was passaged every 3–4 days on media without antibiotic exposure to act as a laboratory passage control. MICs for all resulting isolates were determined by Etest.

Bacterial growth for RNAseq

To obtain the transcriptomic profile of resistant isolates under antibiotic pressure, CIDM-BH3 and CIDM-BPP2 (and their respective resistant descendants) cultures were grown in LB broth in triplicates. A loopful of colonies was transferred to LB broth, homogenized and divided equally into three tubes, the MF standard was calculated, and was kept consistent across treatment conditions. Susceptible (BH3OG and BPP2OG) and resistant isolates were cultured overnight (stopped at 12 h) with aeration at 250 rpm, in liquid media either with erythromycin (256 mg/L) (BH3RAB and BPP2RAB) or without the antibiotic (BH3RES and BPP2RES).

Extraction and sequencing

Genomic DNA was extracted with the DNeasy Blood and Tissue Mini Kit (QIAGEN, Germany) or DNeasy UltraClean Microbial Kit (QIAGEN, Germany) for Illumina and Nanopore sequencing, respectively. WGS was performed at the Microbial Genomics Reference Laboratory, NSW Health Pathology. All strains were short-read sequenced on the NextSeq platform (Illumina, USA). In addition, strains CIDM-BP2, CIDM-BP3, CIDM-BH3, CIDM-BPP2 and CIDM-BPP2R were also long-read sequenced on the MinION platform (Oxford Nanopore Technologies plc, UK). Sequencing libraries for Illumina sequencing were prepared using the Nextera XT DNA Library Prep Kit (Illumina) and sequenced on a NextSeq 500 using NextSeq 500/550 v2 mid output kits (Illumina). Sequencing libraries for Nanopore sequencing were prepared using the Rapid Barcoding kit (SQK-RBK004) and sequencing on a R9 flowcell. Total RNA was extracted from liquid cultures using the RNeasy Plus Universal Mini Kit (QIAGEN, Germany), following manufacturer's protocol.

Total RNA sequencing was performed by the Australian Genomics Research Facility (AGRF) utilizing the Illumina Stranded Total RNA Prep with Ribo-Zero Plus on the NovaSeq.

Genome analysis

The short-read sequenced raw reads were quality controlled using FastQC (v 0.11.3), Trimmomatic (v 0.36)[17] and Centrifuge (v 1.0.4),[18] prior to further analysis. For the strains sequenced by short-read technology, trimmed reads were assembled with default parameters by SPAdes (v 3.12.0).[19] All assemblies were then annotated with Prokka (v 1.12)[20] and Barnapp (v 0.6) (https://github.com/tseemann/barrnap), then scanned for virulence factors (VFDB)[21] and resistance markers (CARD)[22] with Abricate (v 0.9.8; https://github.com/tseemann/abricate). For long-read sequencing, base calling was performed on high accuracy mode and demultiplexing was performed on Guppy (v 2.4.5) (https://github.com/nanoporetech/pyguppyclient) on a GPU Amazon Web Service instance. Demultiplexed reads were then *de novo* assembled with Flye (v 2.7b)[23] with the '–asm-coverage' parameter set to 30 and an expected genome size of 4.0 Mb. Following long-read assembly, the sequence was corrected with Racon (v 1.3.1)[24] four times, and Medaka (v 0.11.5)[24] twice. The assembly was then polished with corresponding Illumina reads using Pilon (v 1.23)[25] and repeated until there were no more changes.

Identification of SNPs in the resistant genomes from organisms that showed increased MICs post-erythromycin induction was performed using Snippy (v 4.3.5) (https://github.com/tseemann/snippy). Reference sequences used were *B. pertussis* Tohama I (NCBI GenBank accession number: NC_002929.2) and the long-read closed genomes or the short-read assembly from this study. Further comparisons of resistance genes between the *Bordetella* spp. were performed by the BLASTn and figures were drawn in EasyFig (v 2.2.2).[26]

Transcriptome analysis

For RNAseq, the raw reads were also passed through the in-house quality control procedure consisting of FastQC (v 0.11.3), Trimmomatic (v 0.36)[17] and Centrifuge (v 1.0.4).[18] Mapping of RNAseq reads onto their corresponding long-read assembled genome was performed using BWA-MEM (v 0.7.17). Closed genomes were initially annotated with the NCBI Prokaryotic Genome Annotation Pipeline,[27–29] and coding sequences were further corrected using EggNOG-mapper.[30,31] HTSeq (v 0.11.2)[32] was used to calculate the number of reads mapped to each gene feature.

Statistical analysis

All read counts were normalized using read counts per million (CPM) and transcripts per million (TPM). Statistical comparisons between treatment conditions were performed using unpaired *t*-tests on GraphPad Prism and plotted using BoxPlotR.[33] Raw data has also been supplied in the Supplementary data.

Data availability

Closed genomes and sequencing reads of resistant isolates have been published in Bioproject: PRJNA224116.

Results

MIC following induction of erythromycin resistance

To induce resistance in four *B. pertussis*, three *B. parapertussis* and three *B. holmesii* strains, isolates were grown on media with an erythromycin Etest or a disc for 15 weeks. *B. parapertussis* isolates gradually increased their MIC levels to those corresponding to *in vitro* resistance (>256 mg/L) within 2 to 7 weeks (within 6–15 passages) (Figure 1 and Figure S2). *B. holmesii* isolates took slightly longer than *B. parapertussis* to develop resistance (>256 mg/L), at 5 to 12 weeks (13–25 passages) (Figure S3). However, after 15 weeks (30 passages), *B. pertussis* did not develop resistance and the MICs of all four isolates fluctuated between 0.032 and 0.38 mg/L (Figure S4).

Genomic variability in isolates with elevated MIC to erythromycin

Passaged isolates were sequenced every month (i.e. 4 weeks/8 passages) to monitor any intermediate genomic variation that may have contributed to phenotypic increase in MIC. For those that developed resistance, the majority of isolates developed the highest resistance within one passage (spontaneously) rather than slowly accumulating resistance and increasing MIC over several passages (progressively), which suggested that resistance was driven by SNPs. Despite some elevation of the MIC of erythromycin, the genomes of the *B. pertussis* and *B. parapertussis* isolates contained no mutations in the 23S rRNA gene sequence reported in macrolide-resistant *B. pertussis*.[5] However, such mutations in the 23S rRNA gene were detected in all resistant *B. holmesii* (Table 3) with each having a distinct mutation in positions G2031A (strain CIDM-BH2), A2032G (CIDM-BH3), and C2585T (CIDM-BH1). Long-read sequencing allowed the differentiation of the three 23S rRNA gene copies, which cannot be resolved with short-read sequencing. Mapping the short-read CIDM-BH3 resistant (CIDM-BH3R) reads to the closed CIDM-BH3 genome confirmed that all three copies of the 23S rRNA carried the A to G mutation in position 2032. No mutations were observed in the 23S rRNA gene for either the CIDM-BPP2 or CIDM-BPP2 resistant strain (CIDM-BPP2R).

As the 23S rRNA gene of CIDM-BPP2R did not possess mutations that were implicated in macrolide resistance, the genome was screened for other genes of interest that could potentially confer resistance to macrolides (Table S1). None of the selected genes was present but analysis against the CARD and VFDB database yielded the presence of the *Pseudomonas aeruginosa mexB* gene. This gene is part of a tripartite efflux pump mechanism described in *P. aeruginosa* and known to confer macrolide resistance.[34] The *mexB* gene was present in both susceptible and resistant strains and BLASTn alignments of the entire *mexAB-oprM* operon yielded 75.6% identity to 88% coverage in both CIDM-BPP2 and CIDM-BPP2R (Figure 2). A BLASTn search of the *mexAB-oprM* operon from the CIDM-BPP2R genome to the entire NCBI nucleotide database, showed that all other *B. parapertussis* strains carried this operon, as did *Bordetella bronchiseptica* with a 99.7% similarity. Thus, presence of this operon in the primary pathogens from the *Bordetella* spp. was determined and is shown in Figure 2.

Transcriptome analysis

With the presence of the *mexAB-oprM* orthologue in *B. parapertussis*, we investigated the changes in transcriptional regulation that could result in macrolide resistance. The whole transcriptome was captured with RNAseq for isolates CIDM-BH3, CIDM-BPP2 and their resistant derivatives. In both cases, three

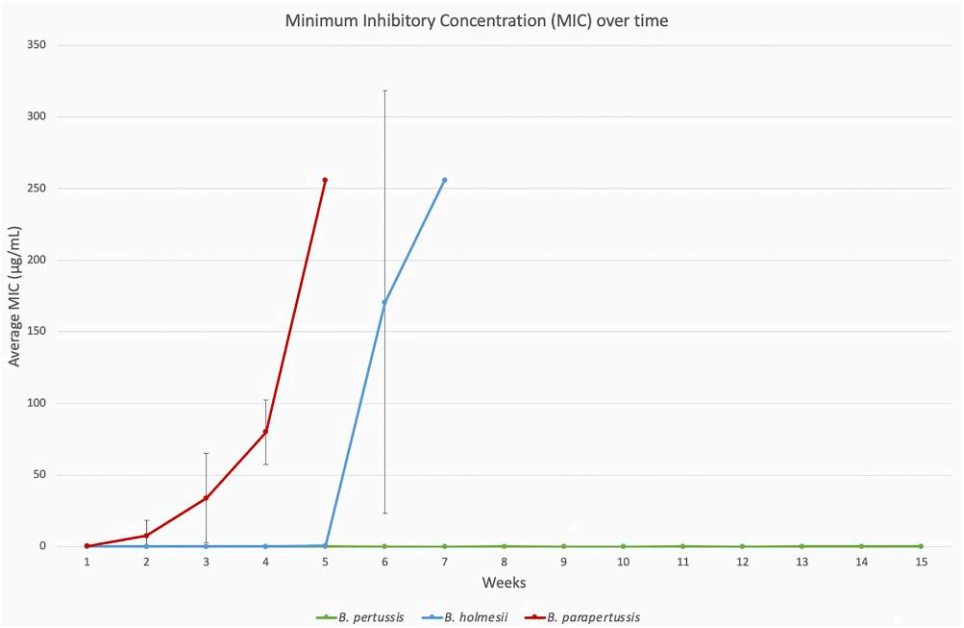

Figure 1. Average erythromycin MIC of *Bordetella* spp. across all 15 weeks. *B. parapertussis* became resistant in 2 weeks (average 4.5 weeks), while *B. holmesii* took on average 6 weeks. However, the erythromycin MIC of *B. pertussis* remained persistently low. Error bars represent the standard deviation of the MIC at the timepoint. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Table 3. Summary of all strains of *Bordetella* spp. enrolled in this study including the initial and final MIC for erythromycin, and presence and absence of the 23S rRNA gene mutation resulting in phenotypic resistance

Sample	Initial MIC (mg/L)	Final MIC (mg/L)	23S rRNA mutations
Bordetella pertussis			
CIDM-BP1	0.032	0.125	Not detected
CIDM-BP2	0.032–0.047	0.125	Not detected
CIDM-BP3	0.016	0.125	Not detected
CIDM-BP4	0.064	0.125	Not detected
Bordetella parapertussis			
CIDM-BPP1	0.125–0.19	>256	Not detected
CIDM-BPP2[a]	1	>256	Not detected
CIDM-BPP3	0.125–0.19	>256	Not detected
Bordetella holmesii			
CIDM-BH1	0.125–0.19	>256	C to T (Position 2585)
CIDM-BH2	0.047–0.064	>256	G to A (Position 2031)
CIDM-BH3	0.25	>256	A to G (Position 2032)

[a]The resistant isolate of CIDM-BPP2 was named CIDM-BPP2R.

growth conditions were applied, the susceptible isolate (CIDM-BH3/BH3OG and CIDM-BPP2/BPP2OG), the resistant isolate (CIDM-BH3R/BH3RES and CIDM-BPP2R/BPP2RES) and the resistant isolate grown under macrolide pressure (BH3RAB and BPP2RAB). The raw TPM data and condition comparisons are provided in the Supplementary data.

The transcriptome profile over the *mexAB-oprM* in CIDM-BPP2 revealed average expression between BPP2OG and BPP2RES also had an average 2.2 ± 0.6-fold upregulation (Figure 3a). However, it was also observed that an average 2.3 ± 0.7-fold upregulation occurred in expression of BPP2RAB compared with BPP2OG (Figure 3b). Further investigations of the transcriptome of *B. parapertussis*, revealed another efflux pump that was highly expressed (i.e. 5.1 ± 1.2-fold increase). This efflux pump was identified to be another *acr*-like pump, named *acr/bepE*, which is closely related to the *mexI/mexW* family of genes in *P. aeruginosa* (Figure S5). The entire gene locus showed a 3.9 ± 3.5-fold increase in expression when BPP2OG was compared with BPP2RAB. However, compared with BPP2RES, it was a 6.3 ± 8.2-fold increase in expression. In addition, the gene within this locus with the

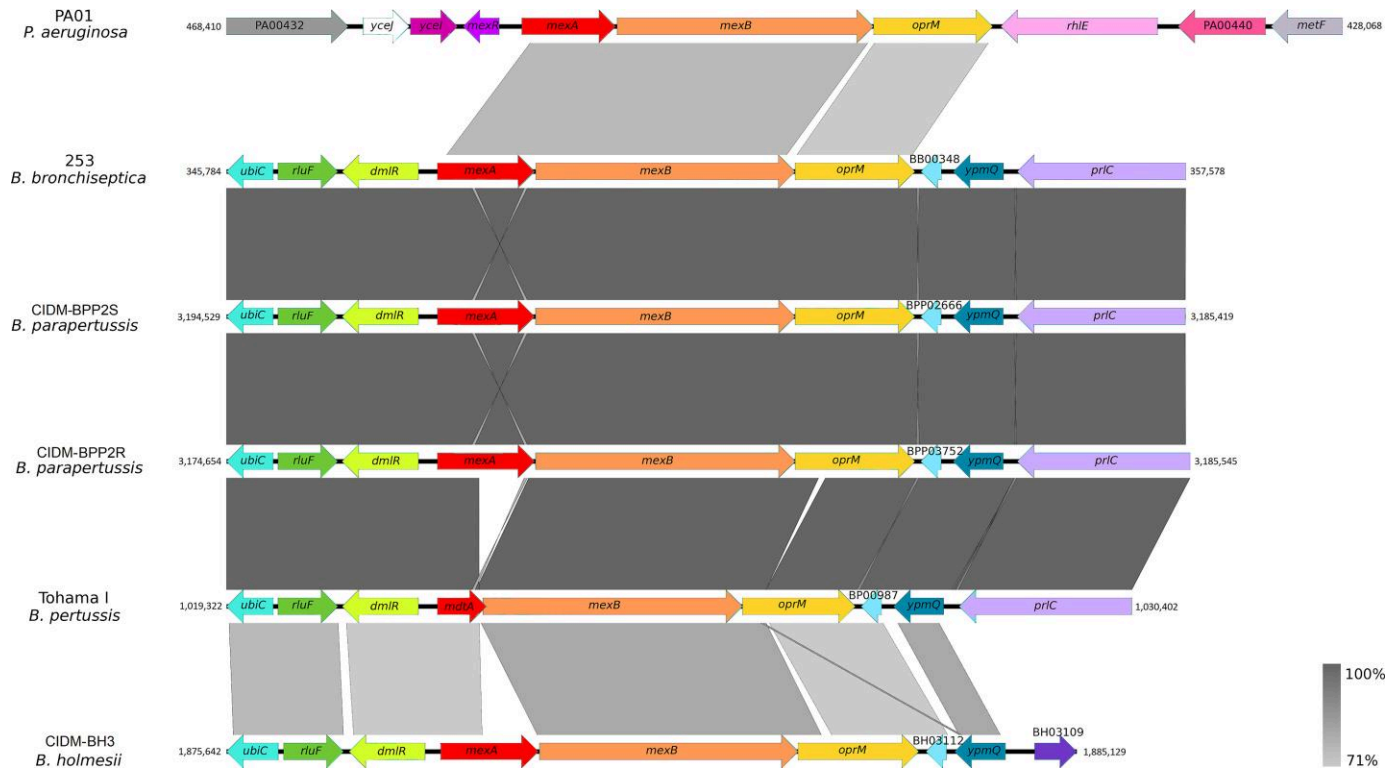

Figure 2. Comparison (BLASTn) of the *mexAB-oprM* operon ±3 flanking genes of *Pseudomonas aeruginosa* PA01 (NC_002516.2), *Bordetella bronchiseptica* 253 (NC_019382.1), *B. parapertussis* CIDM-BPP2 and CIDM-BPP2R, *B. pertussis* Tohama I (NC_002929.2), and *B. holmesii* CIDM-BH3. The primary pathogens from the *Bordetella* spp. carry a >71% orthologue by BLASTn of the *mexAB-oprM* operon, however, there is a gene deletion present in the *mexA* and *oprM* genes in *B. pertussis*. Nucleotide sequence similarity is scaled according to the scale bar. This image was generated using EasyFig.[26] This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

highest increased fold-change (7.4-fold in BPP2RAB and 14.5-fold in BPP2RES) was the efflux transporter outer membrane subunit (*oprN*). Examination upstream and downstream of this operon demonstrated that the first TR (adjacent to *oprN*) is an ArsR family transcriptional regulator, which are repressors of di- and multi-valent heavy metal ions. Adjacent to the TR is a highly upregulated azurin (*azn)* gene, hence the TR and azurin are likely linked (Figure S6).

The transcriptome of *B. holmesii* under antibiotic pressure behaved similarly to that of *B. parapertussis.* The expression profile over the *mexAB-oprM* equivalent locus, revealed an average 1.6 ± 0.6-fold increase in BH3OG versus BH3RAB, and a 1.1 ± 0.3-fold change in upregulation for BH3OG versus BH3RES (Figure 4). Further, the *acr*-like operon present in *B. parapertussis* was not detected in *B. holmesii*.

Resistance mechanism and housekeeping gene expression

To further clarify the changes in expression of housekeeping genes, a set as selected by the *Bordetella* spp. MLST scheme and the *bvgAS* locus were investigated and compared alongside the *mexAB-oprM* operon. Of the seven housekeeping genes, three (*tyrB*, *pepA* and *pgm*) in *B. parapertussis* remained consistently expressed across all normalized conditions (fold-change between 0.9–1.1). However, three were downregulated ∼2-fold (*adk*, *fumC* and *glyA*), and one was upregulated 1.54 ± 0.06-fold (*icd*) (Supplementary data).

Discussion

This study demonstrated that repeated exposure to erythromycin induced *in vitro* resistance in *B. parapertussis* and *B. holmesii* but not in *B. pertussis* for the duration of our study. While exposure decreased susceptibility to erythromycin in *B. pertussis*, the MICs did not reach levels defined as *in vitro* resistance. The predicted mechanisms of resistance varied between species, with *B. holmesii* containing a 23S rRNA gene mutation and *B. parapertussis* having no obvious mutations relating to macrolide resistance in that gene. The *B. holmesii* strains each acquired unique 23S rRNA mutations in different nucleotide positions, all of which have conferred resistance to macrolides in previous reports.[5] As induced erythromycin-resistant *B. parapertussis* did not possess mutations in the 23S rRNA gene, other possible resistance mechanisms such as the presence of *erm*, *mef*, *mex* or *ere* were investigated.

While the 23S rRNA mutation was the most likely explanation for induced resistance in *B. holmesii,* isolates of *B. parapertussis* did not contain mutations in the same region. We found that none of the *erm*, *mef* or *ere* genes were present in the

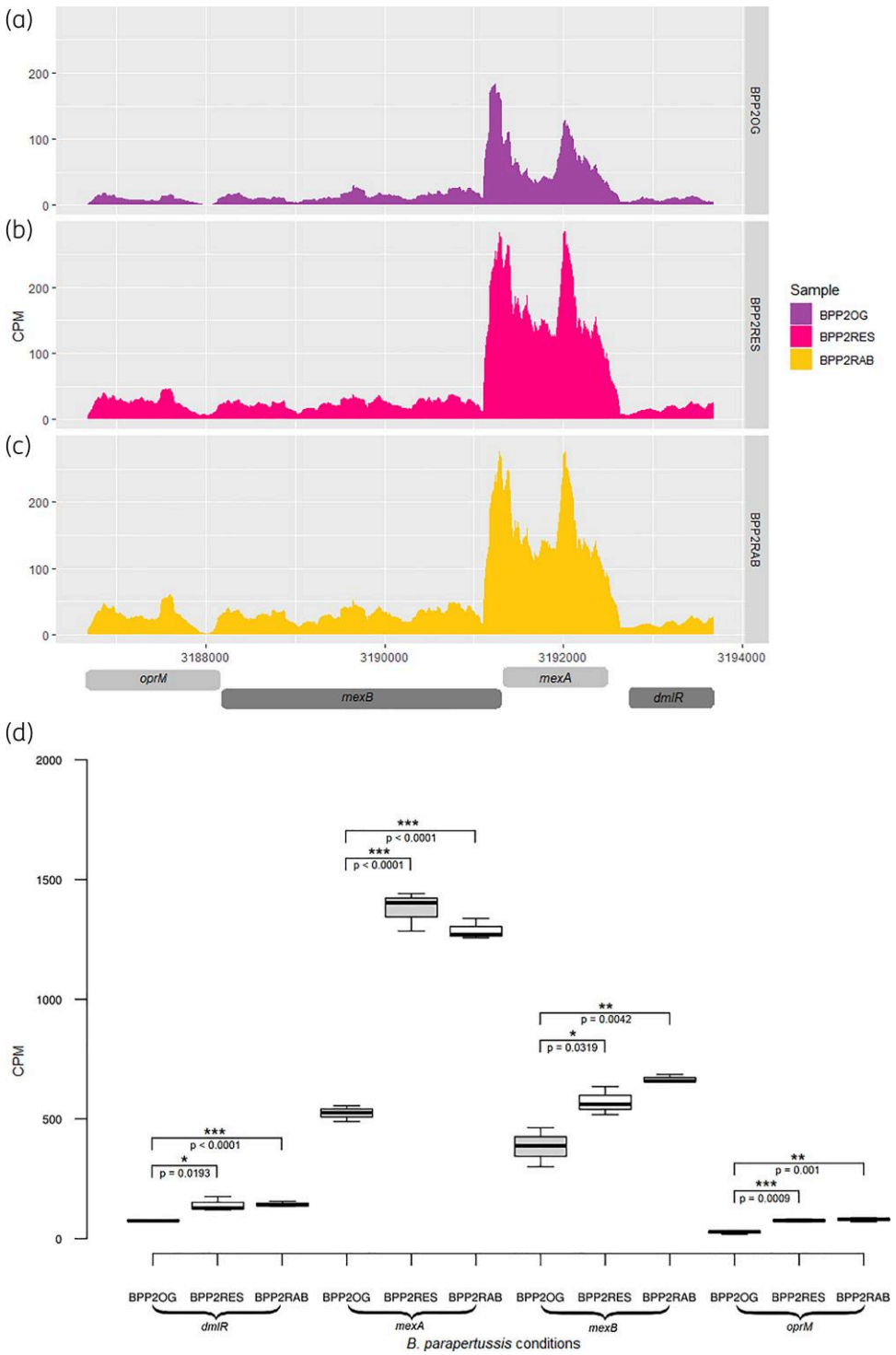

Figure 3. Expression profile of the *mexAB-oprM* orthologue in *B. parapertussis* CIDM-BPP2 calculated based on genomic position and CPM. (a and b) Comparison of BPP2OG (erythromycin susceptible) with BPP2RES (resistant without antibiotic pressure), demonstrating a large proportion of reads encompassing the *mexA* gene. (a and c) Comparison of BPP2OG (susceptible) with BPP2RAB (resistant with antibiotic pressure), shows a similar outcome as BPP2RES. (d) Box plot of gene expression between conditions. For genes within *mexAB-oprM* and its transcriptional regulator (*dmlR*) all were significantly upregulated in the RES and RAB conditions. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

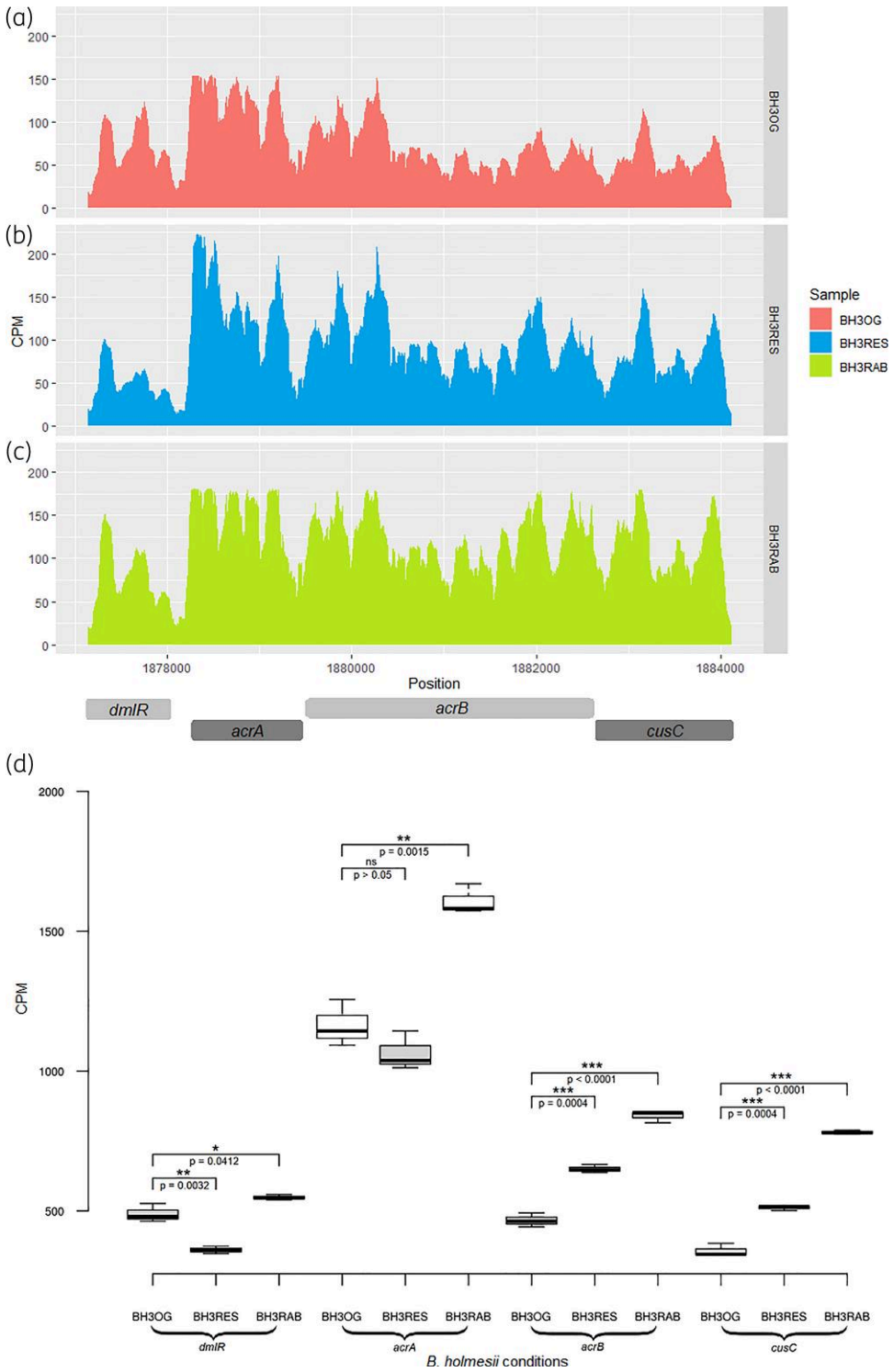

Figure 4. Expression profile of the *mexAB-oprM* equivalent efflux pump in *B. holmesii*. (a and b) Comparison of BH3OG (susceptible) with BH3RES (resistant without antibiotic pressure), demonstrating relatively even distribution of reads across the gene locus. (a and c) Comparison of BPP2OG (susceptible) with BPP2RAB (resistant with antibiotic pressure), showed a similar outcome as BPP2RES. (d) Box plot of gene expression in isolates under different conditions. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Bordetella species, however, an orthologue of the *mexAB-oprM* operon with >71% homology was detected in the genomes of all mammalian *Bordetella* spp. (*B. pertussis*, *B. parapertussis*, *B. holmesii*, and *B. bronchiseptica*). Previous genomic annotation predictions have called the *mexAB-oprM* system the *acrAB-cusC* operon, with the latter conferring resistance to acriflavine, other hydrophobic molecules and fatty acids.[35] As erythromycin is a hydrophobic molecule, the *mexAB-oprM* system could facilitate the excretion of this molecule by the efflux pump system and this may explain the acquisition of induced resistance in this study. The *mexAB-oprM* operon is present in *Pseudomonas aeruginosa* and encodes an efflux pump that confers macrolide resistance. The *mexAB-oprM* operon in *B. parapertussis* had high sequence similarity (99.7%) to *B. bronchiseptica*, and the function of *mexAB-oprM* is predicted to be the same in both species. *B. bronchiseptica*, the common ancestor of *B. parapertussis* and *B. pertussis*,[13,36] is inherently macrolide resistant with an MIC between 4–32 mg/L,[37,38] and is also known to rapidly develop macrolide resistance upon antibiotic pressure.[39]

For *B. pertussis*, the *mexA* and *oprM* genes were considerably different to the orthologues in *B. bronchispetica* and *B. parapertussis*. A deletion of 646 bp in the 3′ region of the *mexA* gene, and an 84 bp in-frame deletion in *oprM* were previously reported in *B. pertussis*.[35] The *B. pertussis mexAB-oprM* operon suffered a reduction in activity due to the two deletions in *mexA* and *oprM*.[40] A previous study by MacArthur *et al.*[35] showed that the transcriptional regulator in both *B. bronchiseptica* and *B. pertussis* for the *mexAB-oprM/acrAB-cusC* operon is *dmlR* (BP0983). In *B. pertussis*, BP0983 sits adjacent to the *mexA* gene, and a deletion of BP0983 has been shown to result in the upregulation of the *mexAB-oprM* operon and depression of transcription in the presence of fatty acids. Similarly, *B. pertussis* isolates in this study shared the same deletion suggesting that our isolates were likely to have increased sensitivity to acriflavine, fatty acids and ampicillin.[35] This could explain the higher susceptibility to erythromycin reported here. *B. holmesii* also carries this orthologue, however it is quite divergent (~80%) from the primary *Bordetella* spp. orthologues. No mutations were detected within the operon region in *B. holmesii*, which suggests the operon is fully functional in this species. The regional deletion of the *mexAB-oprM* operon in *B. pertussis* and full functionality of the operon in *B. holmesii* could explain the elevated MIC in *B. holmesii* (0.047–0.25 mg/L) in comparison with *B. pertussis* (0.016–0.064 mg/L).

To further investigate the role of the *mexAB-oprM* orthologue operon in erythromycin resistance, we performed a transcription study using RNAseq to observe the expression of the operon under antibiotic pressure. In *B. parapertussis*, an ~2-fold upregulation of *mexAB-oprM*, in conjunction with another upregulated *mex-/acr*-like efflux pump was found when *B. parapertussis* was grown in 256 mg/L of erythromycin. While an upregulation of transcription was detected in *mexAB-oprM*, it is difficult to conclude whether this upregulation is the cause of the phenotypic resistance observed. As the *mexAB-oprM* operon in *P. aeruginosa* confers a resistant MIC of >256 mg/L, it is possible that even a slight upregulation (3-fold) in *oprM* would result in changes in susceptibility.[41] However, both the resistant (RES) and resistant strain under antibiotic pressure (RAB) conditions demonstrated an upregulation in these markers, suggesting the change in expression was not a result of direct pressure from antibiotics but a form of constitutive expression developed during antibiotic pressure passages. In *B. holmesii*, the *mexAB-oprM* appears to be regulated consistently, which could further explain the naturally higher MIC. Therefore, whether the *mexAB-oprM* or any other efflux pump is the cause of resistance will require further investigation. In addition, we could not definitively comment if the upregulated efflux pump was a transient response. Further experiments to investigate whether phenotypic resistance persists beyond the time limits of this experiment and whether induced resistance is maintained without antibiotic pressure could help elucidate this point.

In conclusion, our findings have indicated that *B. holmesii* and *B. parapertussis* can readily develop phenotypic resistance to erythromycin under antibiotic pressure, while *B. pertussis* did not in the conditions described here. The predicted mechanisms of resistance varied between species, with *B. holmesii* containing a recognized 23S rRNA gene mutation and *B. parapertussis* having no obvious mutations relating to macrolide resistance. The presence of the *mexAB-oprM* orthologue (*acrAB-cusC* operon) has the potential to confer macrolide resistance in *B. parapertussis*. Genomic data and isolates with induced resistance can serve as reference points for development of diagnostic assays and surveillance of macrolide resistance in *Bordetella* recovered from patients with clinical pertussis.

These findings have significant implications for the development of antibiotic guidelines on treatment and prophylaxis of pertussis caused by these pathogens as infection with *B. parapertussis* or *B. holmesii* can be misdiagnosed as *B. pertussis*. The understanding of mechanisms of macrolide resistance and the ability to detect resistance in a timely fashion can improve patient outcomes and reduce the spread of the disease. The ability of *B. parapertussis* and *B. holmesii* to rapidly acquire macrolide resistance highlights the need for better surveillance and antibiotic stewardship in the management and control of pertussis cases and outbreaks.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Figures S1 to S6 and TPM data are available as Supplementary data at *JAC* Online.

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