

Mutational and Transcriptomic Changes Involved in the Development of Macrolide Resistance in *Campylobacter jejuni*

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Macrolide antibiotics are important for clinical treatment of infections caused by *Campylobacter jejuni*. Development of resistance to this class of antibiotics in *Campylobacter* is a complex process, and the dynamic molecular changes involved in this process remain poorly defined. Multiple lineages of macrolide-resistant mutants were selected by stepwise exposure of *C. jejuni* to escalating doses of erythromycin or tylosin. Mutations in target genes were determined by DNA sequencing, and the dynamic changes in the expression of antibiotic efflux transporters and the transcriptome of *C. jejuni* were examined by real-time reverse transcription-PCR, immunoblotting, and DNA microarray analysis. Multiple types of mutations in ribosomal proteins L4 and L22 occurred early during stepwise selection. On the contrary, the mutations in the 23S rRNA gene, mediating high resistance to macrolides, were observed only in the late-stage mutants. Upregulation of antibiotic efflux genes was observed in the intermediately resistant mutants, and the magnitude of upregulation declined with the occurrence of mutations in the 23S rRNA gene. DNA microarray analysis revealed the differential expression of 265 genes, most of which occurred in the intermediate mutant, including the upregulation of genes encoding ribosomal proteins and the downregulation of genes involved in energy metabolism and motility. These results indicate (i) that mutations in L4 and L22 along with temporal overexpression of antibiotic efflux genes precede and may facilitate the development of high-level macrolide resistance and (ii) that the development of macrolide resistance affects the pathways important for physiology and metabolism in *C. jejuni*, providing an explanation for the reduced fitness of macrolide-resistant *Campylobacter*.

Campylobacter jejuni is a leading cause of diarrhea and one of the most common bacterial causes of food-borne illnesses worldwide (1). Because of the decreasing therapeutic effectiveness of fluoroquinolones, macrolide antibiotics (e.g., erythromycin [Ery]) have become more and more important for the treatment of *Campylobacter* infections in humans (2, 3). Additionally, some macrolide antibiotics, including tylosin (Tyl), Ery, and tilmicosin, are also used in food animal production for both therapeutic and subtherapeutic purposes (4). As a zoonotic pathogen transmitted through the food-borne route, *Campylobacter* is subject to selection pressure from macrolide use in both veterinary medicine and human medicine. Although the overall prevalence of macrolide-resistant *Campylobacter* is relatively low, high incidences of macrolide resistance in *Campylobacter* have been reported in some studies (5–9). In order to curb the emergence of macrolide resistance, it is necessary for us to understand how *Campylobacter* develops resistance to this class of antibiotics.

In *Campylobacter*, macrolide resistance is mediated by target mutations and antibiotic efflux (2). Mutations in the L4 and L22 proteins appear to mediate low (MIC, 8 to 16 $\mu\text{g/ml}$)-to-intermediate (MIC, 16 to 128 $\mu\text{g/ml}$) levels of resistance to Ery, while mutations in the 23S rRNA gene are associated with high-level (MIC, ≥ 256 $\mu\text{g/ml}$) Ery resistance (5, 10, 11). Selection of mutations in the 23S rRNA gene seems to require prolonged exposure to macrolide antibiotics, suggesting that other mutations or changes in *Campylobacter* may be required prior to the occurrence of the 23S rRNA mutations (2, 10). Active efflux via the CmeABC multidrug efflux pump is another mechanism that confers resistance to macrolides on *Campylobacter* (10, 12, 13). By inactivation of the CmeABC efflux pump in resistant *Campylobacter* strains, previous studies revealed that CmeABC functions synergistically

with the mutations in ribosomal proteins L4 (G74D) and L22 (insertions at position 86 or 98) or the 23S rRNA gene (A2075G) to confer macrolide resistance (10, 11, 13–15). In *Campylobacter*, expression of *cmeABC* is controlled by CmeR, which binds to the promoter of *cmeABC* and regulates the expression of the efflux operon (16, 17). In addition to CmeABC, previous studies (12, 18) also suggested that other efflux mechanisms might be associated with Ery resistance, but the identities of the suspected efflux pumps are unknown.

Despite the improved understanding of the mechanisms of macrolide resistance in *Campylobacter*, the molecular processes leading to the generation of stable high-level resistance are largely unknown. Understanding of these molecular events will potentially facilitate the clinical use of antibiotics and targeted control of macrolide-resistant *Campylobacter*. Toward this end, we conducted a systemic analysis of the development of macrolide-resistant mutants in culture medium under selection pressure. Multiple lineages of macrolide-resistant *C. jejuni* mutants were selected *in vitro* by stepwise exposure of *C. jejuni* NCTC 11168 and 81-176

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to increasing concentrations of Ery or Tyl. The occurrence of mutations in the 23S rRNA gene (three copies) and the genes encoding ribosomal proteins L4 and L22 was monitored during this multistep selection process. The dynamic changes in the expression of four antibiotic efflux genes (*cmeB*, *Cj1687*, *Cj1257c*, and *Cj1375* [*cmeG*]) in representative lineages were determined by real-time reverse transcription (RT)-PCR and immunoblotting (for CmeABC only). DNA microarray analysis was also carried out with a representative lineage to analyze the global transcriptional changes that occur during the development of macrolide resistance in *C. jejuni*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. jejuni* strains NCTC 11168 and 81-176 were used as the parent strains for *in vitro* selection of Ery^r and Tyl^r mutants. Both strains were originally isolated from humans, are commonly used for laboratory studies, and have been sequenced (accession no. NC_002163.1 and NC_008787.1). Both strains are susceptible to Ery and Tyl, and the MICs are 1 and 4 µg/ml, respectively. The *Campylobacter* strains were grown in Mueller-Hinton (MH) broth or agar at 42°C under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂).

Susceptibility test. MICs of macrolide antibiotics were measured by the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (19). *C. jejuni* ATCC 33560 was used as the quality control strain.

Stepwise selection of macrolide-resistant mutants *in vitro*. Ery and Tyl were used to select macrolide-resistant mutants *in vitro*. They were chosen because Ery is the drug of choice for treating human *Campylobacter* infections, while Tyl and Ery are macrolide antibiotics commonly used in food animal production for therapeutic and subtherapeutic purposes (4). For the first round of selection, cultures of parent strains *C. jejuni* NCTC 11168 and 81-176 were spread on plates containing a series of increasing concentrations (0.5 × MIC, 1 × MIC, and 2 × MIC) of Ery or Tyl (11). Following 3 to 5 days of incubation under microaerobic conditions at 42°C, single colonies on the plate with the highest concentration of the drugs were randomly selected and used for subsequent stepwise selection. Stepwise selection was performed by transferring and passaging colonies selected in the first round to MH broth with gradually increased drug (Ery or Tyl) concentrations. Briefly, each colony picked in the first-round selection was separately enriched in antibiotic-free MH broth to the late logarithmic phase to a density of 1 × 10⁸ CFU/ml, from which 100 µl (containing approximately 10⁷ CFU) was transferred to 10 ml MH broth containing the same concentration of drugs in the first-round selective plates. After 2 to 3 days of incubation, the cells in each culture were collected as a unit and subjected to MIC determinations. For the next selection step, 100 µl (containing approximately 10⁷ CFU) of the culture from the previous step was inoculated into 10 ml of MH broth containing a 2-fold higher concentration of the antibiotic used in the previous step. During selection with each antibiotic concentration, multiple passages might be needed to allow adaptation and optimal growth of the selected mutants. The selective concentration of the antibiotic was gradually increased for each lineage up to the concentration at which the cultures were not able to grow. A lineage was defined as a series of derivatives selected from a single colony that was obtained in the first-round selection.

PCR amplification and DNA sequence analysis. The primers used to amplify the sequences of the 23S rRNA gene, *rplD* (L4), *rplV* (L22), and *cmeR* along with the promoter of *cmeABC* are listed in Table 1. All three copies of the 23S rRNA gene in each of the mutant strains were amplified and sequenced as described by Gibreel et al. (20). All PCR products were sequenced in the DNA facility at Iowa State University.

RNA extraction and real-time quantitative RT-PCR (qRT-PCR). To prepare RNA, *C. jejuni* cultures were grown on MH plates for 12 h and harvested with MH broth containing RNAProtect Bacterial Reagent (1:2; Qiagen, Valencia, CA). RNA extraction, purification, and quantification

TABLE 1 Primers used for PCR amplification and real-time RT-PCR

Primer	Sequence (5'–3')	Reference
23S rRNA gene amplification		
FI	CCCTAAGTCAAGCCTTTCAATCC	20
FII	CGTTATAGATACGCTTAGCGGTTATG	20
FIII	CATCGAGCAAGAGTTTTATGCAAGC	20
CJ-copy-R	CTACCCACCAGACATTGTCCCAC	20
Fragment of 23S rRNA gene		
23S-domV-F	GTAACCGCGGCCGTAACATA	This study
23S-domV-R	GACCGAAGTGTCTCACGACG	This study
23S-2611F	ATGTCGGCTCATCGCATCTGG	20
23S-2611R	CATCCATTACACACCCAGCCTATC	20
Ribosomal protein L4		
L4-F	GTAGTTAAAGGTGCAGTACCA	This study
L4-R	GCGAAGTTTGAATAACTACG	This study
Ribosomal protein L22		
L22-F	GAATTTGCTCCAACACGC	This study
L22-R	ACCATCTTGATTCCCAGTTTC	This study
CmeR and <i>cmeABC</i> promoter		
cmeR-F	TAGAAAAGTATATTTGTATACCCT	16
cmeR-R	CGCCACTAAGCTTGAGGCTTTA	16
Real-time RT-PCR ^a		
16S-F	TACCTGGGCTTGATATCCTA	21
16S-R	GGACTTAACCCAACATCTCA	21
CmeB-F	ACGATTCAACCTTTTCCCAGC	This study
CmeB-R	TTTGCTACTTGAGCAATCGCTTC	This study
Cj1687-F	TGGTGGTTTTGGACTCTTTCTGGGA	This study
Cj1687-R	TCTCTGCGATTAAAGCCACCACGA	This study
Cj1375-F	GCTTTACCACGATTTTCTTCTGTGA	This study
Cj1375-R	TACACCATGCTTTTAGGAAGAATGC	This study
Cj1257c-F	ATCGCCGTGATAGCGCCTAAAGAA	This study
Cj1257c-R	GCCACCAAACAAAGGCCCAAGTAA	This study

^a Sequences and gene names are based on *C. jejuni* NCTC 11168.

were performed as previously described (21). The extracted RNA was further treated with an On-Column DNase Digestion kit (Qiagen), followed by an additional DNase treatment to remove residual DNA contamination. The prepared RNA samples were used as the template for real-time qRT-PCR and microarray analysis. The relative expression of four antibiotic efflux transporter genes (*cmeB*, *Cj1687*, *Cj1257c*, and *Cj1375*) in four representative lineages (68Ex-1, 68Ex-3, 76Ex-6, and 76Tx-7) was determined by qRT-PCR, which was performed with the Bio-Rad IQ thermocycler and the iScript One-Step RT-PCR kit with SYBR green as described previously (21). The primer sets used for specific genes are listed in Table 1. 16S rRNA was used as a control for normalization (22).

SDS-PAGE and immunoblotting. Whole-cell proteins of various *Campylobacter* strains were separated by SDS-PAGE and immunoblotted using anti-CmeA, anti-CmeB, and anti-CmeC antibodies as described previously (23).

DNA microarray analysis and data analysis. Three Ery^r mutants (68E1-3, 68E8-3, and 68E64-3) in lineage 68Ex-3 were analyzed by microarray analysis in comparison with *C. jejuni* NCTC 11168. The microarray analysis slides of *C. jejuni* (version 1) were obtained from the J. Craig

TABLE 2 Characteristics of *C. jejuni* mutants selected by Ery

Lineage ^a and strain ^b	MIC ($\mu\text{g/ml}$)		Mutation in 23S rRNA gene ^c	Change in protein L4	Change(s) in protein L22	Mutation in <i>cmeR</i>
	Ery	Tyl				
Parent, NCTC 11168	1	4	— ^d	—	—	—
68Ex-1						
68E1-1	4	8	—	—	A88E	—
68E4-1	32	256	—	—	A88E	Del490A
68E8-1	64	128	—	—	A88E	Del490A
68E32-1	256	128	—	G67V	A88E	Del490A
68E64-1	>512	256	C2627A (3)	G67V	A88E	Del490A
68Ex-2						
68E1-2	4	8	—	59-G-60	—	—
68E4-2	32	128	—	59-G-60	G86E	—
68E8-2	64	128	—	59-G-60	A84D, G86E	—
68E32-2	128	128	—	59-G-60	A84D, G86V	—
68E64-2	512	256	—	59-G-60	A84D, G86V	—
68Ex-268Ex-3						
68E1-3	4	16	—	R72I	—	—
68E4-3	64	64	—	R72I	V100L	H174N
68E8-3	128	256	—	R72I	91-S-92	H174N
68E32-3	>512	>512	—	R72I	—	H174N
68E64-3	>512	>512	A2074C (3)	R72I	—	H174N
Parent, 81176	1	4	—	—	—	—
68Ex-268Ex-376Ex-6						
76E2-6	32	64	—	—	—	—
76E8-6	256	>512	A2074C (2)	—	—	—
76E64-6	>512	>512	A2074C (3)	—	—	—

^a The lineages are named according to the parent strain and the antibiotic used for selection. 68 and 76 indicate that the parent strains are 11168 and 81-176, respectively, E represents selection by Ery, x depicts the various concentrations used for selection, and the last number indicates the lineage number.

^b The strains in the lineages are named similarly to the lineages, except that x is replaced by the actual antibiotic concentration from which the mutant was selected.

^c Each number in parentheses is the number of mutated copies of the 23S rRNA gene.

^d —, no mutations detected.

Venter Institute. For each microarray analysis slide, a pair of cDNA samples prepared from NCTC 11168 and a mutant culture, respectively, were cohybridized. Four hybridizations using four independent RNA samples were performed for each pair of *C. jejuni* cultures. cDNA labeling, preparation, and hybridization were performed following the protocols from the J. Craig Venter Institute. Data collection and normalization were performed as previously described (17). After statistical analysis, the set of *p* values was converted to *q* values to control the false-discovery rate. For this study, genes with a *q* value of <0.1 and a change of ≥ 1.5 -fold were identified as being differentially expressed by the resistant and wild-type strains. Differentially expressed genes were first classified on the basis of the genomic annotation at the Sanger Center and then subjected to clustering analysis (K-means/medians clustering [KMC]) and hierarchical clustering (HCL) analysis using Multiexperiment Viewer (MeV v4.4).

Microarray data accession number. The microarray data obtained in this study have been deposited in the NCBI Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/database>) and assigned accession number GSE17881.

RESULTS

Lineages of the selected macrolide-resistant mutants. Three individual colonies (68E-1, 68E-2, and 68E-3) of parent strain *C. jejuni* NCTC 11168 were randomly selected on plates containing 1 $\mu\text{g/ml}$ Ery in the first-round selection and subjected to stepwise selection to obtain three representative lineages of Ery^r mutants

(68Ex-1, 68Ex-2, and 68Ex-3). Similarly, one representative colony (76E-6) of parent strain *C. jejuni* 81-176 was selected on a plate with 2 $\mu\text{g/ml}$ Ery in the first-round selection and one lineage of Ery^r mutants (76Ex-6) was obtained after stepwise selection. The characteristic features of the four lineages are described in Table 2.

By using Tyl as the selective agent, four individual colonies (68T-1, 68T-2, 68T-3, and 68T-4) of parent strain *C. jejuni* NCTC 11168 were selected on plates with 4 $\mu\text{g/ml}$ Tyl and one colony (76T-7) of *C. jejuni* 81-176 was selected on plates with 8 $\mu\text{g/ml}$ Tyl in the first round of selection. After stepwise selection, five representative lineages of Tyl^r mutants (68Tx-1, 68Tx-2, 68Tx-3, 68Tx-4, and 76Tx-7) were obtained; their characteristic features are listed in Table 3.

Dynamic changes in point mutations in target genes and proteins. As shown in Tables 2 and 3, multiple mutations were observed in the target genes (the 23S rRNA gene, the genes for ribosomal proteins L4 and L22, and *cmeR*). Overall, the mutations in the genes for L4 and L22 occurred earlier in the stepwise selection process, while the mutations in the 23S rRNA gene occurred later in the selection process and were associated with high-level resistance to Ery (MIC, ≥ 256 $\mu\text{g/ml}$).

Six different changes in ribosomal protein L4 were observed in

TABLE 3 Characteristics of *C. jejuni* mutants selected by Tyl

Lineage ^a and strain ^b	MIC (μg/ml)		Mutation in 23S rRNA gene	Change in protein L4	Change(s) in protein L22	Mutation in <i>cmeR</i>
	Ery	Tyl				
Parent, NCTC 11168	1	4	— ^c	—	—	—
68Tx-1						
68T4-1	4	8	—	—	A88E	—
68T16-1	32	256	—	R72I	A88E	H174N
68T32-1	64	256	—	R72I	A88E	H174N
68T128-1	128	256	—	R72I	A88E	H174N
68Tx-2						
68T4-2	4	8	—	—	—	—
68T16-2	128	256	—	G57V	—	—
68T32-2	256	512	—	G57V	G86E	—
68T128-2	128	256	—	G57V	G86E	—
68 Tx-3						
68T4-3	2	8	—	—	—	—
68T16-3	32	256	—	A71D	—	S117Y
68T32-3	64	512	—	A71D	A88E	S117Y
68T64-3	16	256	—	A71D	A88E	S117Y
68Tx-4						
68T4-4	2	8	—	—	—	—
68T8-4	16	64	—	A71D	—	—
68T16-4	32	128	—	A71D	A88E	G204L
68T32-4	64	256	—	A71D	A88E	G204L
68T64-4	128	512	—	A71D	A88E, G86V	G204L
Parent, 81176	1	4	—	—	—	—
76Tx-7						
76T8-7	8	32	—	—	—	—
76T16-7	32	128	—	G57D	G86E	—
76T32-7	64	256	—	G57D	G86E	—

^a See Table 2 footnote *a* for an explanation of the lineage names. T represents selection by Tyl.

^b See Table 2 footnote *b* for an explanation of the strain names.

^c —, no mutations detected.

the macrolide-resistant mutants selected (Tables 2 and 3). The Gly-to-Val (G57V) and Gly-to-Asp (G57D) modifications identified at position 57 in L4 were previously reported by Caldwell et al. (11). Four other mutations in ribosomal protein L4, including R72I, G67V, and A71D and the insertion of a glycine at position 59, represent new mutations identified in this study. In a given lineage, only one type of mutation was observed in L4.

Modifications at positions 84, 86, and 88 were frequently observed mutations in ribosomal protein L22 in the macrolide-resistant mutants (Tables 2 and 3). G86E and A88E were more frequent than other types of mutations in the mutants analyzed. In lineage 68Ex-2, the G86E mutation in L22 was switched to G86V and the modification at position 86 coexisted with A84D (Table 2). With respect to the 23S rRNA gene, the A2074C mutation was observed in the high-level Ery^r mutants of two lineages (68Ex-3 and 76Ex-6). In 68E64-3, the A2074C change was present in all three copies of the 23S rRNA gene. In the 76Ex-6 lineage, the A2074C transversion occurred earlier in the mutant selection process. This point mutation was present in only two of the three copies of the 23S rRNA gene in 76E8-6 but in all three copies of the 23S rRNA gene in 76E64-6 (Table 2). Interestingly, the high-level

Ery^r mutant (68E64-1) in lineage 68Ex-1 displayed a different type of point mutation in the 23S rRNA gene with a C→A point mutation at position 2627 (equivalent to position 2611 in *Escherichia coli*). In one of the lineages selected with Ery (68Ex-2), stepwise selection failed to select mutants containing mutations in the 23S rRNA gene (Table 2), while none of the Tyl-selected mutants displayed point mutations in the 23S rRNA gene (Table 3).

Dynamic changes in the expression of antibiotic efflux genes.

The qRT-PCR results (Fig. 1) showed that there was a general trend for increased expression of the four efflux genes (*cmeB*, *Cj1687*, *Cj1257c*, and *Cj1375*) in the lineages examined (68Ex-1, 68Ex-3, 76Ex-6, and 76Tx-7). In lineage 68Ex-1 (Fig. 1B), the most obvious change was a drastic increase in the expression of *cmeB* and *Cj1687* in 68E4-1, and the magnitudes of overexpression of the two efflux genes subsequently declined in the later-stage mutants of the lineage. In the 68Ex-3 lineage (Fig. 1A), all four of the efflux genes tested were upregulated and the magnitudes of upregulation of *cmeB*, *Cj1257c*, and *Cj1687* were greater than that of *Cj1375*. In lineage 76Ex-6 (Fig. 1C), overexpression of *cmeB* was observed in 76E2-6 and 76E8-6, while the expression level of *cmeB* in 76E64-6 returned to the level of the parent strain,

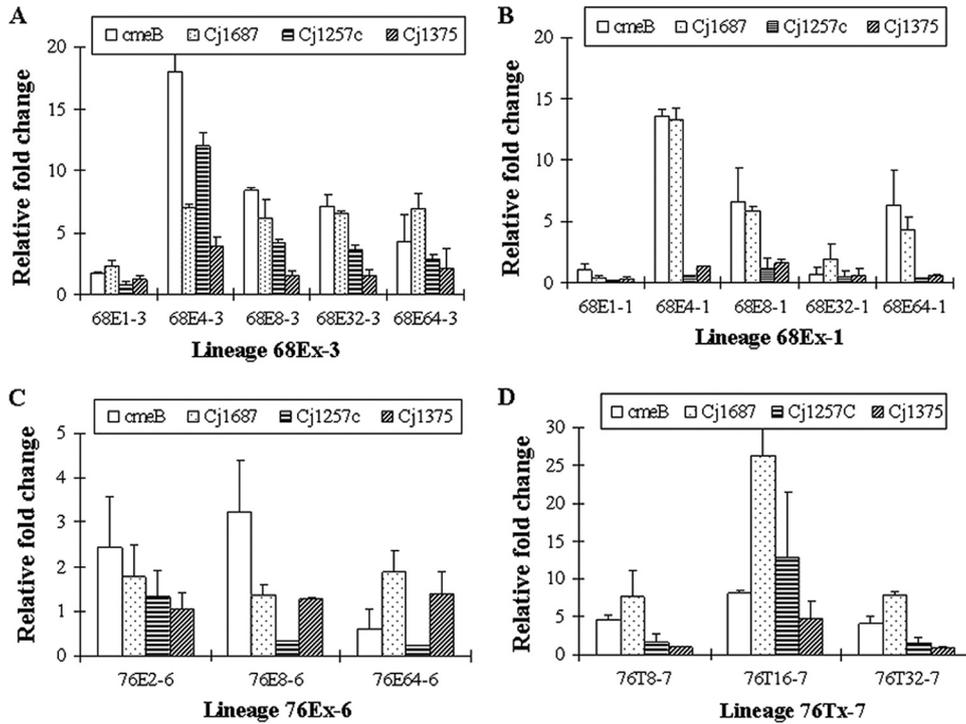


FIG 1 Differential expression of four efflux genes in selected mutant strains in four different lineages. The data were generated by real-time RT-PCR in comparison with the parent strain (NCTC 11168 or 81-176).

C. jejuni 81-176. In lineage 76Tx-7 (Fig. 1D), *cmeB* and *Cj1687* were significantly overexpressed and the change was especially obvious for *Cj1687* in 76T16-7. Overall, there was a general trend for the overexpression of the efflux genes to be temporarily up-

regulated in the mutants with intermediate-level resistance and subsequently decline in the later-stage mutants.

Immunoblotting analysis further showed the change in Cme-ABC expression in the lineages (Fig. 2). In each lineage, the pro-

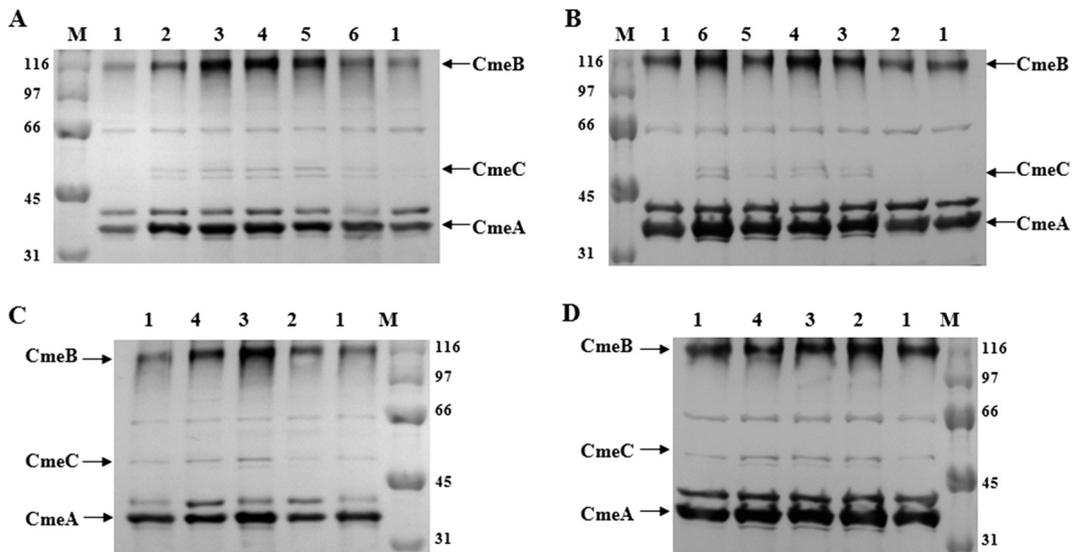


FIG 2 Immunoblotting analysis of CmeABC expression in selected strains in four different lineages. In panel A, numbers 1, 2, 3, 4, 5, and 6 represent *C. jejuni* NCTC 11168, 68E1-3, 68E4-3, 68E8-3, 68E32-3, and 68E64-3, respectively. In panel B, numbers 1, 2, 3, 4, 5, and 6 correspond to *C. jejuni* NCTC 11168, 68E1-1, 68E4-1, 68E8-1, 68E32-1, and 68E64-1. In panel C, numbers 1, 2, 3, and 4 correspond to *C. jejuni* 81-176, 76E2-6, 76E8-6, and 76E64-6. In panel D, numbers 1, 2, 3, and 4 correspond to *C. jejuni* 81-176, 76T8-7, 76T16-7, and 76T32-7. Cell envelopes prepared from each strain were blotted with polyclonal antibodies to CmeA, CmeB, and CmeC. The same amount of total proteins was loaded in each lane. Prestained molecular mass markers (lane M; Bio-Rad) were used to estimate the sizes of the proteins (shown in kilodaltons). The positions of CmeA, CmeB, and CmeC are indicated.

duction of CmeABC proteins increased during the mutant selection process. For example, the CmeB protein was significantly overproduced in 68E4-3, 68E8-3, and 68E32-3 (lanes 3, 4, and 5 in Fig. 2A) compared with the wild-type strain. Likewise, a significant increase in CmeB production was observed in 76E8-6 (lane 3 in Fig. 2C).

Identification of mutations in *cmeR*. Since *cmeABC* was overexpressed in the mutant selection process (Fig. 1 and 2), we further determined if any mutations occurred in *cmeR* and the promoter of *cmeABC* that might explain the overexpression of *cmeABC*. Three different types of point mutations (H174N, S117Y, and G204L) and a deletion (del490A) were detected in *cmeR* in some mutant strains of five different lineages, including 68Ex-1, 68Ex-3, 68Tx-1, 68Tx-3, and 68Tx-4 (Tables 2 and 3). The del490A nucleotide deletion would cause a frameshift and truncation of the CmeR open reading frame, which may explain the overexpression of *cmeB* in lineage 68Ex-1 (Fig. 1 and 2). The H174N substitution in CmeR also occurred concomitantly with the observed overexpression of *cmeB* in lineage 68Ex-3, suggesting that the H174N mutation may affect the function of CmeR. No mutations in the promoter of *cmeABC* were observed in any of the Ery^r mutants.

Dynamic changes in the transcriptome during mutant selection. To examine the global change at the transcriptional level in the mutant selection process, three Ery^r mutants (68E1-3, 68E8-3, and 68E64-3) of lineage 68Ex-3 were subjected to microarray analysis in comparison with *C. jejuni* NCTC 11168. The transcriptional abundance of 265 genes was altered in at least one of the three strains. In 68E1-3, only nine genes were found differentially expressed in comparison with the parent strain. However, in 68E8-3, 85 genes were upregulated and 124 genes were downregulated, and in 68E64-3, 52 genes were upregulated and 93 genes were downregulated (see Fig. S1 and Table S1 in the supplemental material). Of the 265 genes, 86 were also changed in 68E8-3 and 68E64-3 (see Fig. S1 and Table S1). Only five genes showed differential expression in all three mutant strains, of which *Cj0561c*, *Cj0365c* (*cmeC*), and *peb3* were upregulated while *Cj1465* and *Cj1357c* were downregulated (see Table S1).

After KMC analysis, the 265 genes were grouped into four clusters, numbered 1, 2, 3, and 4 (Fig. 3). The clusters clearly visualized the trends of transcriptional changes during the development of macrolide resistance in *C. jejuni*. Cluster 1 includes 64 genes the expression of which was not changed in 68E1-3 but upregulated in 68E8-3; and then in 68E64-3, their overexpression was reduced, with many returning to a near-wild-type level. On the contrary, the 122 genes in cluster 2 were downregulated in 68E8-3 but the magnitudes of downregulation were reduced in 68E64-3. Cluster 3 includes 40 genes whose expression level continuously increased in the lineage as macrolide resistance increased. The 39 genes included in cluster 4 showed little change in expression in 68E1-3 and 68E8-3 but were downregulated in highly resistant strain 68E64-3. Additionally, from the order or distance of the three strains exhibited in the HCL-Tree (Fig. 3) in each cluster, it can be seen that the differentially expressed genes in 68E8-3 are characteristic changes in clusters 1 and 2, while the transcriptomic alterations in 68E64-3 better represent the characteristic changes in clusters 3 and 4.

The 64 genes in cluster 1 include those encoding broad regulatory functions (*perR*, *cetB*, and *cbrR*), efflux pumps (*Cj1173*, *cmeB*, *cmeC*, and *Cj0035c*), ribosomal proteins (*rpmC*, *rpsCEJ*, and *rpl-CDEMW*), aminoacyl tRNA synthetases (*fmt* and *cysS*), DNA rep-

lication/protein translation-associated proteins (*gidA*, *ligA*, *def*, and *pnp*), and small-molecule metabolism (*tkf*, *hisL*, *ilvE*, *nrdB*, *bioB*, *panBD*, and *fabF*). Additionally, the cluster includes genes encoding membrane proteins (*Cj1170c*, *Cj0378c*, and *Cj0124c*), surface polysaccharides (*Cj1425c*, *Cj1430c*, and *lpxB*), periplasmic proteins (*Cj0561c*, *Cj1169c*, *Cj0515*, and *Cj1021c*), ABC transporters (*livHJG* and *Cj0469*), a molybdenum transport system (*modBC*), and amino acid transporters, as well as other transporters (*Cj0982c*, *Cj0025c*, *Cj1389*, and *Cj1097*).

Cluster 2 includes genes encoding broad regulators (*Cj0448* and *Cj1491c*), ribosomal proteins (*rplI* and *rpsA*), membrane proteins (*Cj1493c*, *Cj1026c*, *pal*, *Cj0423*, *Cj0176c*, *porA*, and *Cj0830*), surface polysaccharides (*Cj1423c* and *kpsM*), flagellar proteins (*flgBCGG₂EE₂KI*, *fliDE*, and *flaABCDG*), nutrient transport/binding proteins (*peb1A*, *chuAD*, *corA*, *cfrA*, *lctP*, *dcuA*, *kgtP*, *Cj0486*, *Cj0654c*, and *exbB2D2*), chaperons or heat shock proteins (*clpB*, *cbpA*, *hrcA*, *dnaK*, *grpE*, and *groEL*), and detoxification enzymes (*ahpC* and *Cj0358*). Additionally, the cluster includes genes involved in carbon compound degradation (*galU* and *pta*), energy metabolism (*pyk*, *gltA*, *sdhAB*, *acnB*, and *frdABC*), respiration (*gpsA*, *Cj1357c*, *Cj1358c*, *Cj0074c*, *Cj0075c*, *Cj0265c*, *fdxA*, and *napAGH*), central intermediary metabolism (*ppa*, *gltB*, *aspA*, and *uxaA*), amino acid biosynthesis (*glnA*, *argG*, *serA*, *aroQ*, and *leuB*), nucleoside biosynthesis (*purD* and *dut*), and fatty acid biosynthesis (*Cj1537c*).

Many of the 40 genes in cluster 3 are involved in protein synthesis, including those for ribosomal proteins (*rplAJTLFPRNVXO* and *rpsDPNSQ*) and elongation factor Tu (*tuf*). Additionally, the cluster includes genes for a transcriptional regulator (*Cj0440c*), membrane proteins (*Cj1658*, *Cj0544*, and *Cj1013c*), a surface polysaccharide (*Cj1136*), miscellaneous periplasmic proteins (*Cj0420*, *Cj1540*, and *peb3*), and cation transporter/binding proteins (*ceuE* and *Cj0175c*), as well as genes involved in energy metabolism (*oorC*, *Cj1153*, and *atpCDE*), amino acid biosynthesis (*metEY*), and peptide secretion (*secY* and *lspA*).

Cluster 4 includes genes encoding a transcriptional regulator (*Cj1000*), transport/binding proteins (*Cj0012c*, *Cj0045c*, *pstC*, and *tonB2*), chaperons or heat shock proteins (*groES* and *hspR*), and a chemotaxis histidine kinase (*cheA*). Additionally, the cluster includes genes involved in peptide secretion (*Cj1471c*), energy metabolism (*sdhC*, *gpsA*, *Cj0037*, *Cj0874c*, and *atpB*), central intermediary metabolism (*ppk* and *cysQ*), amino acid biosynthesis (*argG* and *trpF*), nucleoside biosynthesis (*pryG* and *upp*), aminoacyl tRNA synthesis (*glyS*), DNA replication (*mutY*), and the biosynthesis of membrane or surface structures (*Cj0629*, *Cj0341c*, *Cj0455c*, *Cj1316c*, *neuB2C2*, *fliA*, *Cj1275c*, and *Cj0004c*).

Several representative differentially expressed genes (*Cj0561c*, *Cj1199*, *Cj1013c*, *flgI*, and *sdhA*) identified by DNA microarray analysis were further confirmed by qRT-PCR. In most of the cases, the qRT-PCR data confirmed the up- or downregulation detected by microarray analysis (data not shown). The relative *n*-fold changes in *Cj0561c* and *Cj1013c* detected by qRT-PCR were much greater than those detected by microarray analysis (data not shown).

DISCUSSION

In this study, the dynamic changes in target gene mutations, expression of antibiotic efflux genes, and the global transcriptome were analyzed during the process of macrolide resistance development in *Campylobacter*. The findings provide a glimpse into the

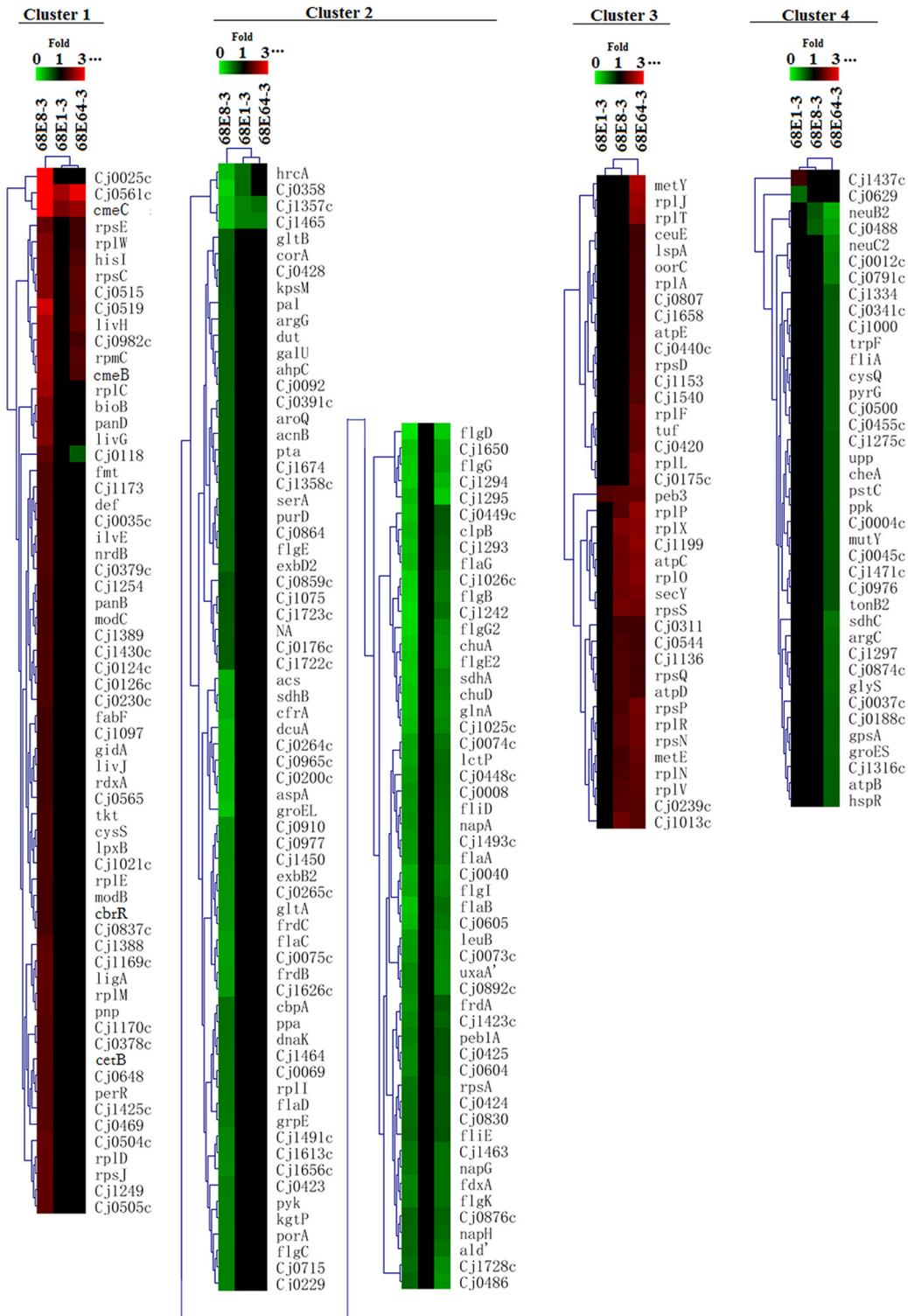


FIG 3 HCL analysis of *C. jejuni* genes differentially expressed in the three strains (68E1-3, 68E8-3, and 68E64-3) in lineage 68E-X. For each of the four clusters, hierarchical clustering was performed both on the genes and on the strains to visualize the relationships among the genes and strains, respectively. Four clusters are presented in four different panels. For each cluster, the color scale shows the n -fold change range of up- and down-regulated genes. The three mutant strains are indicated at the top of each panel.

development of macrolide resistance in *C. jejuni*, which involves mutations in L4 and L22 preceding the occurrence of mutations in the 23S rRNA gene, temporal upregulation of multidrug efflux genes, and transcriptomic changes in metabolic pathways and cel-

lular processes. These results indicate a complex adaptive process involved in the development of macrolide resistance in *Campylobacter*.

It is known that modifications in the large loop of the L4 pro-

tein (amino acids [aa] 55 to 77) (24, 25) and in the highly conserved large loop of the L22 protein (aa 78 to 98) (26, 27) were associated with macrolide resistance in various bacteria. In this study, several mutations in ribosomal proteins L4 (R72I, G67V, A71D, and the insertion of a glycine at position 60) and L22 (A84D, G86E, G86V, and A88E) were observed in macrolide-resistant mutants (Tables 2 and 3), indicating that mutations in the ribosomal proteins are actively involved in the adaptation to macrolide selection in *Campylobacter*. It appears that some modifications in ribosomal proteins L4 (R72I) and L22 (A88E and G86E) may facilitate the development of high-level resistance to macrolides in *Campylobacter*, because they were frequently observed in derivatives with different levels of macrolide resistance (5). However, some mutations in L4, such as G57D, G57V, and A71D, seem to impede the emergence of highly resistant mutants (mutations in the 23S rRNA gene), because subsequent selection of mutants carrying such mutations did not further increase the Ery MIC (Table 3). A similar phenomenon was observed in previous studies (11, 28). These observations suggest that mutations in L4 and L22 are commonly involved in the development of macrolide resistance, but certain mutations in the ribosomal proteins might be incompatible with the mutations in the 23S rRNA gene, preventing the emergence of highly resistant mutants.

Interestingly, there were some differences in the mutations selected with Tyl and Ery. The mutations G57D, G57V, and A71D in L4 were observed only in mutants selected with Tyl (Tables 2 and 3). On the other hand, the mutations in the 23S rRNA gene occurred only in mutants selected with Ery, and selection with Tyl was not able to obtain mutants highly resistant to Ery (MIC, ≥ 512). A previous study also found that Tyl failed to select *Campylobacter* mutants that harbor mutations in the 23S rRNA gene in culture medium (11). However, Ery^r mutants that harbored mutations in the 23S rRNA gene developed in *C. jejuni*-infected chickens fed a growth-promoting dose of Tyl for a prolonged time (10). These observations suggest that the selection environment influences the development of macrolide-resistant mutants. Additionally, Ery is a 14-membered macrolide while Tyl is a 16-membered macrolide with slightly different ribosome binding sites (29). Thus, the structural variations between the two antibiotics might also contribute to the differences in the mutants selected.

C. jejuni has three copies of the *rrm* operon (20, 30, 31). Multiple studies have identified Ery^r mutants (Ery MIC, >64 $\mu\text{g/ml}$) that carried the A2074C or A2075G mutation in two or three copies of the 23S rRNA gene (11, 20, 28, 32, 33). A previous study reported that the A2074G mutation in only one copy of the 23S rRNA gene resulted in a moderate increase in the Ery MIC (8 $\mu\text{g/ml}$) for a *Campylobacter coli* mutant that was selected with Ery (10). In this study, the A2074C transversion was observed in at least two copies of the 23S rRNA gene (Table 2). In lineage 76Ex-6, further selection of the mutant with the A2074C transversion in two copies of the 23S rRNA gene led to the occurrence of the same mutation in all three copies and a concurrent increase in the macrolide MICs (Table 2). Together, these observations suggest that the number of mutated copies of the 23S rRNA gene increasingly reduces the susceptibility of *Campylobacter* to macrolide antibiotics but a mutation in at least two copies of the 23S rRNA gene is required for high-level resistance to macrolides.

In *Campylobacter*, the frequency of emergence of single-step macrolide-resistant mutants is quite low ($<10^{-9}$), and *de novo*

development of macrolide-resistant mutants requires prolonged exposure to the antibiotic (10), suggesting that multiple mutations are required to raise the MICs of macrolide antibiotics. During the stepwise selection process in this study, 10^7 CFU of the organisms were inoculated into 10 ml of MH broth with an elevated antibiotic concentration. This inoculum was well below the number required for the presence of preexisting multiple mutations. Thus, multiple passages were required during certain selection steps to allow the selection of new mutations that support full growth at an elevated antibiotic concentration. Despite the difference between this methodology and the model of selection in an animal host (10), the evolutionary pathways appear to be similar, i.e., involving mutations in L4 and L22, followed by the occurrence of mutations in the 23S rRNA gene. It should be pointed out that this study measured mutations in only a few target genes. Mutations in other chromosomal genes likely also contribute to the development of macrolide resistance in *Campylobacter* and were not measured in this work.

The overexpression of CmeABC in the stepwise selection process was consistently shown with three techniques, including qRT-PCR, immunoblotting, and microarray analysis (Fig. 1, 2 and 3), suggesting that the differential expression of this predominant antibiotic efflux system facilitates the development of macrolide resistance. Besides *cmeABC*, two major facilitator superfamily (MFS) transporters (*Cj1687* and *Cj1257c*) were particularly upregulated as detected by qRT-PCR (Fig. 1), while the microarray analysis result revealed the significant upregulation of a small multidrug resistance (SMR) superfamily member transporter (*Cj1173*) and an MFS transporter (*Cj0035c*) in 68E8-3 (Fig. 3, cluster 1). Although a previous study (34) found that insertional inactivation of *Cj1687*, *Cj1257c*, *Cj1174*, and *Cj0035c* did not alter the susceptibility of *C. jejuni* to Ery, the upregulation of these efflux transporters during stepwise selection suggests that they may play a role in the development of macrolide resistance. *Cj1375* (*cmeG*) is another MFS transporter of *C. jejuni* and was shown to confer resistance to antibiotics and oxidative stress; however, overexpression of *cmeG* did not increase resistance to Ery (35). In this study, *cmeG* was upregulated in some lineages but the change was not as obvious as that in other efflux genes examined.

An interesting observation of this study is that the overexpression of the antibiotic efflux genes was most significant in the intermediately resistant mutants and the level of overexpression actually declined in the late-stage mutants (Fig. 1, 2, and 3). This finding suggests that the enhanced expression of the efflux genes is temporarily required to facilitate the development of highly resistant mutants, in which mutations in the 23S rRNA gene or other changes stabilize the resistance phenotype. The interplay of the antibiotic efflux pump CmeABC and ribosomal protein L4/L22 was also observed in previous studies in which it was found that the efflux system acted synergistically with some modifications in ribosomal proteins L4 and L22 to mediate intermediate-level macrolide resistance and facilitate the development of mutation in the 23S rRNA gene (14, 15). Together, these observations clearly indicate that the dynamic change in efflux gene expression interacts with target mutations to facilitate *Campylobacter* adaptation to the selection pressure from macrolide treatment.

In *Campylobacter*, expression of *cmeABC* is controlled by CmeR, which functions as a repressor of *cmeABC* (16). The overexpression of *cmeABC* during stepwise selection could be partly

explained by mutations in CmeR, such as the nucleotide deletion at 490A and the H174N change in the protein (Tables 2 and 3). On the basis of the sequence and crystal structure of CmeR, these mutations are located in the C-terminal domain of CmeR (36–39). The deletion at 490A is expected to truncate the CmeR protein, which may explain the significant upregulation of *cmeABC* in the 68Ex-1 lineage (Table 2; Fig. 1B and 2B). The H174 residue of CmeR is in the ligand-binding pocket of CmeR and is important for the interaction with various ligands, including bile acid, glycerol, and drugs (38, 39). Thus, the H174N mutation likely affects the function of CmeR and leads to the upregulation of *cmeABC* in the 68Ex-3 lineage (Table 2; Fig. 1A and 2A). This notion is further supported by the fact that *Cj0561c* was also upregulated in lineage 68Ex-3 (Fig. 3). *Cj0561c* encodes a putative periplasmic protein and is subject to direct regulation by CmeR (17). Thus, overexpression of *Cj0561c* further suggested that the H174N mutation altered the function of CmeR. The direct impact of the CmeR mutations on the function of CmeR remains to be determined in future studies.

It should be pointed out that the overexpression of *cmeABC* cannot be totally explained by mutations in CmeR, as some lineages (such as 76Ex-6 and 76Tx-7) did not harbor any mutations in CmeR (or the promoter of *cmeABC*) but showed overexpression of *cmeABC* (Tables 2 and 3; Fig. 1 and 2). Additionally, although the same CmeR mutation (such as in lineages 68Ex-1 and 68Ex-3) existed in the late-stage mutants, the levels of *cmeABC* overexpression declined in these highly resistant mutants. These observations suggest that a CmeR-independent mechanism also modulates the expression of *cmeABC*. Indeed, a recent study indicated that CosR, an oxidative stress regulator, serve as the second repressor of *cmeABC* (40). Thus, any mutations that affect the function of CosR would potentially modulate the expression of *cmeABC*. It is also likely that the accumulation of other mutations offsets the need for *cmeABC* overexpression in late-stage mutants. These possibilities await further examination in future studies.

The microarray analysis produced several interesting findings. First, few genes were differentially expressed in the first-step mutant, while most of the differential gene expression was observed in the intermediate-level-resistant mutant (68E8-3) and the number of differentially expressed genes and the magnitudes of differential expression were significantly reduced in highly resistant strain 68E64-3, which contained the A2074C mutation in all three copies of the 23S rRNA gene. This finding strongly suggests that global changes at the transcriptomic level are temporarily involved in the development of macrolide-resistant mutants and that there is a tendency to restore the transcriptome to the wild-type state in the stable highly resistant mutant. Second, a large number of genes encoding ribosomal proteins (*rpIJPTXOLRFNVAWCDME*, *rpsPNSCDQEJ*, and *rpmC*) were upregulated. Macrolides target the bacterial ribosome and inhibit protein synthesis, and the resistance-associated mutations in L4 and L22 alleviate the inhibitory effect of the antibiotics but might reduce the efficiency of protein synthesis (5). Thus, upregulation of the protein synthesis machinery is likely a compensatory response to the reduced rate of protein synthesis in the mutants. Third, a large number of genes involved in the heat shock response, motility, and energy metabolism were significantly downregulated in the macrolide-resistant mutants, suggesting that the development of macrolide resistance in *C. jejuni* profoundly impacts *Campylobacter* physiology and may result in a growth burden and fitness cost. Indeed,

several recent studies found that macrolide-resistant *Campylobacter* shows a significant fitness cost *in vitro* and *in vivo* and is out-competed by susceptible *Campylobacter* in the chicken host in the absence of antibiotic selection pressure (41–44).

In summary, this study has revealed dynamic changes in target gene mutations and differential gene expression during the stepwise selection of macrolide resistance in *Campylobacter*. Particularly, the results discovered a temporal overexpression of antibiotic efflux pumps in the selection process, which interacts with mutations in L4 and L22 and facilitates the development of stable and highly resistant mutants. Additionally, the study also revealed that there is an intermediate “chaos” state in terms of global gene expression in the development of macrolide resistance and further adaptation to antibiotic selection appears to favor the return of the transcriptome to the wild-type state in highly resistant mutants. These findings depict a complex process involving interplay between target mutations and differential gene expression and provide new insights into the molecular mechanisms underlying the development of macrolide resistance in *C. jejuni*.

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