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Synergy between Efflux Pump CmeABC and Modifications in Ribosomal Proteins L4 and L22 in Conferring Macrolide Resistance in *Campylobacter jejuni* and *Campylobacter coli*[▽]

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Macrolide-resistant mutants of *Campylobacter jejuni* and *Campylobacter coli* were selected in vitro using erythromycin and tylosin. These mutants exhibited modifications in the ribosomal proteins L4 (G74D) and L22 (insertions at position 86 or 98). A synergy between the CmeABC efflux pump and these modifications in conferring macrolide resistance was observed.

The zoonotic microorganism *Campylobacter* is a leading cause of human diarrheal disease (9). Macrolides are, with fluoroquinolones, the drugs of choice for treatment of these infections. However, macrolide resistance, although less frequent than fluoroquinolone resistance, was recently reported to increase in clinical strains in several countries (1, 15).

Macrolides and related molecules inhibit protein synthesis by binding to the vicinity of the peptidyl transferase center (14, 17). Three mechanisms of resistance have been described: drug inactivation, active efflux, and modification of the target sites by methylation or mutation (16, 18). Changes in the ribosomal proteins L4 and L22 were associated with clinical resistance to macrolides-lincosamides-streptogramins-ketolides in several bacteria (14, 16).

In *Campylobacter*, mutations in the 23S rRNA genes and efflux, mediated by the CmeABC pump, were both shown to contribute to macrolide resistance (11). Mutations in L4 and L22 have been investigated recently by Corcoran and coworkers (3) and Gibreel et al. (6), but no macrolide resistance-associated alteration was found in these ribosomal proteins.

In this work, spontaneous macrolide-resistant mutants were obtained by plating bacteria on increasing concentrations of erythromycin and tylosin (0.75- to 4-fold initial MIC of the strain). Two strains were used: *Campylobacter jejuni* NCTC 11168 and *Campylobacter coli* C342 (isolated from poultry by the Agence Française de Sécurité Sanitaire des Aliments [Ploufragan, France]).

MICs of resistant clones were determined by the agar dilution method as described previously (12) and compared to those of parental strains. MIC breakpoints used were those recommended by the French Antibiogram Committee (available at http://www.sfm.asso.fr/). For the resistant clones, the MICs of erythromycin and tylosin were 8- to 64-fold higher than the initial MICs for the parental strains (Table 1). The MIC of the fluoroquinolone ciprofloxacin was not modified (Table 1), nor were the MICs of chloramphenicol (data not shown).

The sequence of domain V of the 23S rRNA genes was analyzed in the resistant clones using primers described in Table 2. No modification was observed (Table 1). Analysis was extended to the *rplD* and *rplV* genes encoding the L4 and L22 ribosomal proteins. The sequences of the specific primers used for PCR amplification are given in Table 2. A G-to-A transition was found at nucleotide 221 of the *rplD* gene sequence in the macrolide-resistant clones obtained with erythromycin as selecting agent. This led to a Gly-to-Asp modification at position 74 of the L4 protein sequence (Table 1; Fig. 1). No modification of the L4 protein sequence was observed in the mutants selected using tylosin. Instead a 9 (ACTTCACAT)- to 12 (GCAAGAGCTAGA)-base tandem duplication at positions 292 and 256 in the *rplV* gene was seen for the C342Tyl16 and 11168Tyl48 mutants, respectively. This led to a 3- to 4-amino-acid insertion at position 98 or 86 of the L22 protein sequence (Table 1; Fig. 1).

To further analyze the resistance pattern conferred by the L4 and L22 modifications, antibiograms were performed using 14 (erythromycin and clarithromycin)-, 15 (azithromycin)-, and 16 (spiramycin)-membered macrolides as well as a ketolide (telithromycin), lincosamides (lincomycin and clindamycin), and an oxazolidinone (linezolid). Antibiograms were performed using Neo-sensitabs (Eurobio, Les Ulis, France) according to the recommendations of the manufacturer for fastidious bacteria. The 14- and 15-membered macrolides and telithromycin were the most affected molecules compared to the 16-membered spiramycin and lincosamides (with a significant decrease seen only with the TSH insertion in the L22 protein). The inhibitory diameter of linezolid was not affected by the L4 and L22 modifications.

Inactivation of the *cmeB* multidrug transporter gene in the resistant mutants was obtained by natural transformation of the strains with genomic DNA (1 μg) of an 81176 *cmeB*::*kan* mutant using the biphasic method as described previously (2). *cmeB* inactivation led to a restoration of the susceptibility of the strains whatever their initial level of resistance (Table 1). Efflux thus plays a key role and is needed concomitantly with L4 or L22 alterations to enable resistance of the bacterium. This was also observed in *Haemophilus influenzae* (13) and is

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TABLE 1. Phenotypic and genotypic data for the isolates and laboratory-created mutants[c]

Strain	MIC (μg/ml) of drug[a]:			Change in protein[b]:	
	ERY	TYL	CIP	L4	L22[d]
Isolates					
11168	1	4	≤0.125		
C342	4	16	16		
Laboratory-created mutants					
11168 *cmeB*::*kan*	0.25	0.5	≤0.125		
11168Ery6	16	32	0.25	G74D	
11168Ery6 *cmeB*::*kan*	≤0.125	0.5	0.25	G74D	
11168Tyl48	64	128	0.125		ins $_{86}$ARAR
11168Tyl48 *cmeB*::*kan*	≤0.125	0.5	≤0.125		ins $_{86}$ARAR
C342 *cmeB*::*kan*	0.25	0.5	1		
C342Ery4	32	64	8	G74D	
C342Ery4 *cmeB*::*kan*	≤0.125	0.5	1	G74D	
C342Tyl16	256	512	16		ins $_{98}$TSH
C342Tyl16 *cmeB*::*kan*	0.25	0.5	1		ins $_{98}$TSH
Transformants					
11168$^{11168Ery6/L4}$	16	32	≤0.125	G74D	
11168$^{11168Tyl48/L22}$	32	64	≤0.125		ins $_{86}$ARAR
C342$^{C342Ery4/L4}$	128	256	16	G74D	
C342$^{C342Tyl16/L22}$	256	512	16		ins $_{98}$TSH

[a] Abbreviations: ERY, erythromycin; TYL, tylosin; CIP, ciprofloxacin.
[b] According to *Campylobacter* sequence numbering.
[c] There were no mutations in 23S rRNA.
[d] ins, insertion.

similar to the interplay already described between efflux and 23S rRNA mutations (2).

Transformation experiments using *rplD* and *rplV* mutated amplicons were undertaken to confirm that the mutations observed in the in vitro-selected mutants confer macrolide resistance. L4 and L22 modifications were successfully transferred to the NCTC 11168 and C342 susceptible strains. The transformants obtained exhibited resistance levels with MICs of erythromycin and tylosin similar to the MICs of the in vitro-selected mutants whereas the MIC of ciprofloxacin was not affected (Table 1).

Forty-three field strains (5 of *C. jejuni* and 38 of *C. coli*) with different levels of resistance to erythromycin were analyzed for the presence of mutations in the *rplD* and *rplV* genes by single-strand conformational polymorphism. Primers were designed from nucleotide sequences of the *C. jejuni* NCTC 11168 and *C. coli* RM 2228 strains (Table 2). A different pattern of migration was observed in a few strains (data not shown). The

TABLE 2. Primers used for PCR amplification and sequencing

Primer	Sequence (5′-3′)	Positions
23S rRNA domain V		
Cj23SrRNAFwd	5′-GTAAACGGCGGCCGTAACTA-3′	1913–1932[a]
23S Rev	5′-CATCCATTACACACCCAGCC-3′	2837–2855[a]
Ribosomal protein L4		
L4 Fwd	5′-GTAGTTAAAGGTGCAGTACCA-3′	1619846–1619866[b]
L4 Rev	5′-GCGAAGTTTGAATAACTACG-3′	1619100–1619119[b]
SSCP L4 Fwd	5′-AGAGCAAATACAGCTCATAC-3′	127–146[c]
SSCP L4 Rev	5′-GTTTCTTTCATTTGTTGGACC-3′	265–285[c]
Ribosomal protein L22		
L22 Fwd	5′-GAATTTGCTCCAACACGC-3′	1617842–1617859[b]
L22 Rev	5′-ACCATCTTGATTCCCAGTTTC-3′	1617292–1617312[b]
L22 CJ SSCP Fwd	5′-TGGTGGCTTTGAAGCAAACG-3′	168–187[d]
L22 CJ SSCP Rev	5′-GCTACTGTTTTTTTCTCTTCAG-3′	326–347[d]
L22 CC SSCP Fwd	5′-CGGCGGTTTTGAAGCGAACG-3′	168–187[e]
L22 CC SSCP Rev	5′-GCTACTGCTTTTTTTGCTTCAG-3′	326–347[e]

[a] Nucleotide position according to the 23S rRNA gene sequence of the *C. jejuni* NCTC 11168 genome (GenBank accession number NC_002163, loci Cjr02-Cjr05-Cjr08) (10).
[b] Nucleotide position according to *C. jejuni* NCTC 11168 complete genome (GenBank accession number NC_002163).
[c] Numbering based on the Cj1706c gene sequence of the *C. jejuni* NCTC 11168 genome.
[d] Numbering based on the Cj1702c gene sequence of the *C. jejuni* NCTC 11168 genome.
[e] Numbering based on the CCO1823 gene sequence of the *C. coli* RM2228 genome (GenBank accession number NZ_AAFL01000012) (5).

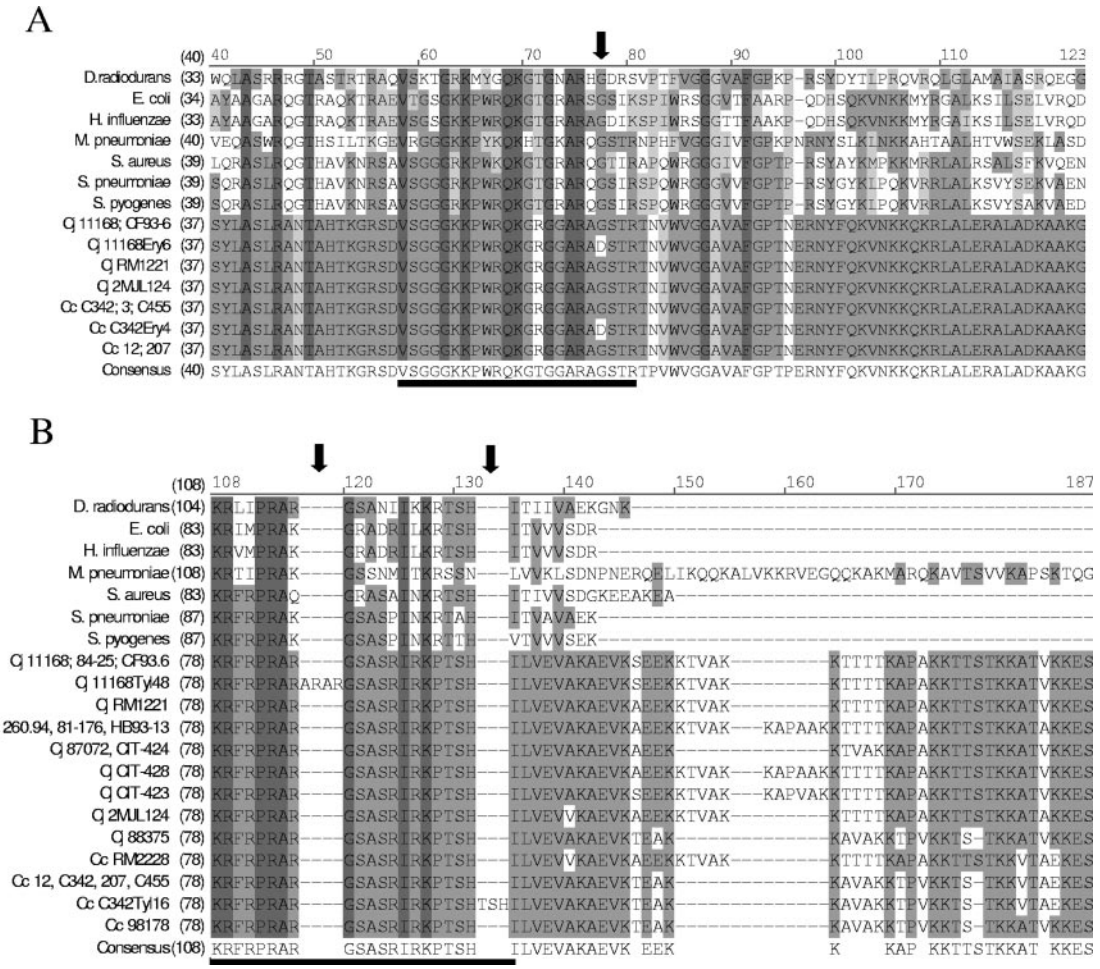

FIG. 1. Comparison of the L4 (A) and L22 (B) protein sequences of *Campylobacter* and other bacterial species: *D. radiodurans*, *Deinococcus radiodurans* R1 (NP_294035 and NP_294039); *E. coli*, *Escherichia coli* K-12 (AAC76344 and AAC76340); *H. influenzae*, *Haemophilus influenzae* Rd KW20 (NP_438937 and NP_438941); *M. pneumoniae*, *Mycoplasma pneumoniae* M129 (NP_109854 and NP_109858); *S. aureus*, *Staphylococcus aureus* MRSA252 (CAG41315 and CAG41311); *S. pneumoniae*, *Streptococcus pneumoniae* R6 (AAK98993 and NP_357788); *S. pyogenes*, *Streptococcus pyogenes* M1 group A streptococcus (AAK33183 and AAK33187); Cj, *Campylobacter jejuni* 11168, NCTC 11168 (CAB73692 and CAB73688), RM1221 (YP_179844 and YP_179840), CF93-6 (ZP_01067485 and EAQ57344), 81-176 (ZP_01087492), 260-94 (ZP-01070430), 84-25 (EAQ95418), HB93-13 (ZP_01070671), 87072 (AAY88727), 88375 (AAY88729), CIT-423 (AAY88725), CIT-424 (AAY88726), CIT-428 (AAZ14851); Cc, *Campylobacter coli* RM2228 (ZP-00370771), 98178 (AAY88728). Accession numbers in parentheses are given respectively for the L4 and L22 protein sequences of each bacterium (or for the L22 protein alone if no sequence of the L4 protein is available). Accession numbers of newly deposited sequences appear in the text. The arrows indicate the modifications occurring in the in vitro-selected mutants. The most conserved residues are underlined. Nucleotide and amino acid alignments were generated using the Vector NTI software Suite 9 (Informax, Frederick, MD). Strains with identical protein sequences appear on the same line.

corresponding genes (five *rplD* and four *rplV* amplicons) were thus amplified, sequenced, and compared to other sequences found in GenBank. All the field strains examined exhibited a T-to-C transition at nucleotide 587 leading to a V196A modification in the L4 sequence. This modification was also found in 13 out of the 18 strains examined by Corcoran et al. (3), in susceptible or resistant strains. One *rplD* amplicon showed a mutation at nucleotide 238 (leading to a V80I protein modification), and two had a mutation at position 82 (P28S change in the protein sequence). The G74D modification found in the in vitro mutants is located in a large loop (region 55 to 77), conserved among all the L4 proteins examined, which is suggested to be the main anchor of this ribosomal protein to 23S rRNA (7). Many modifications in this loop of the L4 protein have been associated with macrolide resistance in other bacteria (14, 16) (Fig. 1). In contrast, the L22-encoding sequences examined showed more changes particularly in the C-terminal region (amino acids 109 to 142) (Fig. 1). The L22 modifications observed in the in vitro-selected mutants of *Campylobacter* are located in a highly conserved large loop (region 78 to 98), corresponding to a β-hairpin of constant length in all bacterial species. Insertions and deletions in this loop have been associated with macrolide resistance in many bacteria (4, 8).

This study describes modifications in the L4 and L22 ribosomal proteins acting in synergy with the CmeABC efflux pump to confer macrolide resistance in in vitro-selected mutants of *C. jejuni* and *C. coli*. A search of modifications in these ribosomal proteins in field strains could thus give a

new insight into the mechanisms of macrolide resistance in *Campylobacter*.

Nucleotide sequence accession numbers. The gene sequences determined in this study were deposited in GenBank under accession numbers DQ639754 (strain 3, *rplD*), DQ639752 and DQ639759 (strain 12, *rplD* and *rplV* genes, respectively), DQ639753 and DQ639760 (strain 207, *rplD* and *rplV* genes, respectively), DQ639755 and DQ639761 (strain C342, *rplD* and *rplV* genes, respectively), DQ639756 and DQ639758 (strain C455, *rplD* and *rplV* genes, respectively), and DQ639757 and DQ639762 (strain 2MJL124, *rplD* and *rplV* genes, respectively).

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