

1                    **A highly macrolide-resistant *Campylobacter jejuni* strain**  
2                    **with rare A2074T mutations in 23S rRNA genes**

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13                    Running title: Characterization of A2074T 23S rRNA mutation in *C. jejuni*

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22           The main molecular mechanisms underlying high-level macrolide resistance in  
23 *Campylobacter* species are multiple mutations such as A2074G, A2074C, and A2075G,  
24 in 23S rRNA genes (1). *Campylobacter* possesses 3 copies of 23S rRNA genes (2) and  
25 the occurrence of two such mutations among 3 copies of 23S rRNA genes has been  
26 reported to confer macrolide resistance (3-5). The association between A2074T  
27 mutations and high-level macrolide resistance in *Campylobacter* has not been fully  
28 elucidated because isolates with these mutations only in some copies of the 23S rRNA  
29 genes have been recovered (6). It was reported that the A2074T mutation might confer  
30 only a low level of macrolide resistance (1, 6). Here, we characterized a highly  
31 macrolide-resistant *Campylobacter jejuni* clinical isolate containing A2074T mutations  
32 in all 3 copies of 23S rRNA genes.

33           The *C. jejuni* strain, NC05-27, was isolated from a patient who had diarrhea  
34 and demonstrated high-level macrolide resistance (Table 1)(7). Classical PCR using  
35 primers that amplify each of the three 23S rRNA genes (Table S1) and Sanger  
36 sequencing analyses in addition to whole genome sequence data (accession no.  
37 BCNK01000000) from MiSeq platforms revealed that this strain possessed a A2074T  
38 mutation in all 3 copies (4). However, this strain possessed no other known resistance  
39 factors, such as the 23S rRNA methyltransferase gene *ermB*, or amino acid changes in  
40 L4/L22 ribosomal proteins (8-10). Therefore, it is strongly suggested that the A2074T  
41 mutations in all 3 copies of 23S rRNA genes were mainly responsible for high-level  
42 macrolide resistance in NC05-27.

43           To further clarify the role of A2074T mutations in macrolide resistance, we

44 PCR-amplified the A2074T mutation-containing 23S rRNA genes from NC05-27  
45 (using primers 23SunivF and 23SunivR [Table S1]) and introduced them into a  
46 macrolide-susceptible *C. jejuni* NCTC11168 strain (Ery<sup>S</sup>) by natural transformation.  
47 The transformants were selected on Mueller-Hinton agar plates supplemented with 64  
48 µg/ml erythromycin. One representative transformant (Ery<sup>R</sup>), confirmed to carry  
49 A2074T mutations in all 3 copies of 23S rRNA gene, was subjected to susceptibility  
50 testing. Ery<sup>R</sup> was found to acquire a high level of macrolide resistance, comparable to  
51 the parent strain NC05-27 (Table 1). Our results indicated that A2074T mutations  
52 conferred high-level macrolide resistance on *Campylobacter* when present in all 3  
53 copies of 23S rRNA genes, along with A2074G, A2074C, and A2075G mutations.

54 The A2074G, A2074C, and A2075G mutations in 23S rRNA genes have been  
55 associated with growth disadvantage, the so-called fitness cost (8, 11, 12). To  
56 understand the influence of A2074T mutations on growth kinetics, isogenic Ery<sup>R</sup> and  
57 Ery<sup>S</sup> strains were grown separately and their proliferation was monitored (Fig. 1A).  
58 There was no considerable difference in the growth rates between Ery<sup>R</sup> and Ery<sup>S</sup> strains.  
59 However, when the strains were evaluated in a mixed culture medium (12), the  
60 Ery<sup>R</sup>/Ery<sup>S</sup> ratio dramatically reduced with increasing number of passages (Fig. 1B).  
61 After three sequential passages, the ratio was below 3%. Consequently, the A2074T  
62 mutations resulted in a growth disadvantage in *Campylobacter*. Although the reason for  
63 the fitness cost caused by the A2074T mutations in 23S rRNA genes is unclear, it is  
64 speculated that the mutations would affect protein synthesis, leading to a fitness burden  
65 under antibiotic-free conditions.

66           We concluded that the A2074T mutations conferred high-level macrolide  
67 resistance when present in all 3 copies of 23S rRNA genes; however, it imposed a  
68 fitness cost in the bacteria.

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124 **Figure legends**

125 FIG. 1. (A) Growth kinetics of Ery<sup>R</sup> and Ery<sup>S</sup> strains. The experiment was repeated  
126 three times. (B) Growth competition assay of Ery<sup>R</sup> and Ery<sup>S</sup> strains in a mixed culture.  
127 The Ery<sup>R</sup>/Ery<sup>S</sup> ratio was initially adjusted to approximately 1:1. At every passage, an  
128 aliquot of cultured broth was diluted and spread on Mueller-Hinton agar plate with and  
129 without 16 µg/ml erythromycin. The number of colonies was counted to estimate the  
130 ratio of Ery<sup>S</sup> and Ery<sup>R</sup> strains. The experiment was repeated four times.

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**TABLE 1.** Susceptibility testing of *C. jejuni* strains to various antibiotics.

Antimicrobial agents	MIC ( $\mu\text{g/ml}$ )		
	<i>C. jejuni</i> NC05-27	<i>C. jejuni</i> NCTC11168 Ery <sup>R</sup>	<i>C. jejuni</i> NCTC11168 Ery <sup>S</sup>
erythromycin	>512	>512	2
clarithromycin	512	512	4
azithromycin	>512	>512	0.25
spiramycin	256	256	4
leucomycin	64	32	0.25
tylosin	>512	>512	8
clindamycin	512	512	1
ciprofloxacin	4	$\leq 0.13$	$\leq 0.13$
tetracycline	1	$\leq 0.13$	$\leq 0.13$
chloramphenicol	8	2	2

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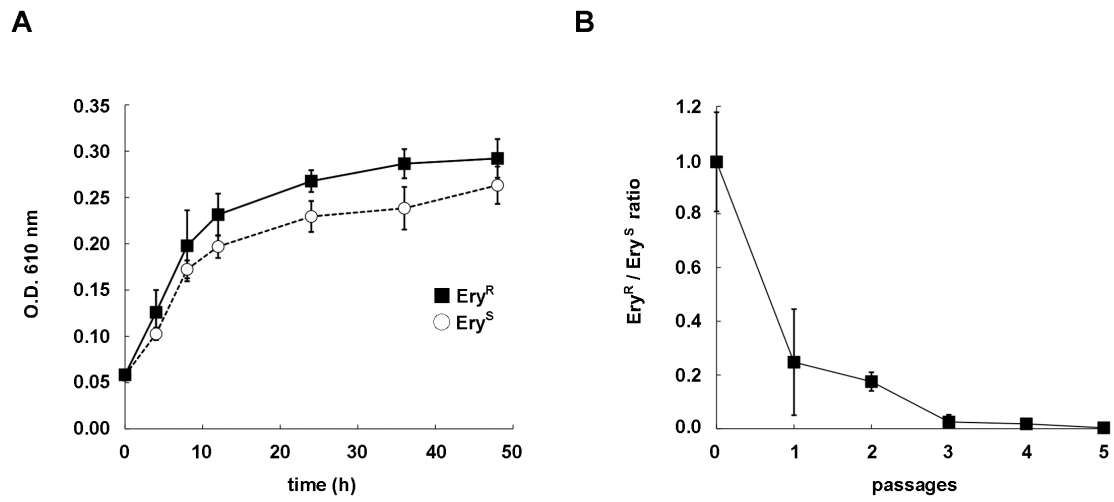
**Figure 1.**

FIG. 1. (A) Growth kinetics of Ery<sup>R</sup> and Ery<sup>S</sup> strains. The experiment was repeated three times. (B) Growth competition assay of Ery<sup>R</sup> and Ery<sup>S</sup> strains in a mixed culture. The Ery<sup>R</sup>/Ery<sup>S</sup> ratio was initially adjusted to approximately 1:1. At every passage, an aliquot of cultured broth was diluted and spread on Mueller-Hinton agar plate with and without 16  $\mu$ g/ml erythromycin. The number of colonies was counted to estimate the ratio of Ery<sup>S</sup> and Ery<sup>R</sup> strains. The experiment was repeated four times.