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Reduced Expression of the *vca0421* Gene of *Vibrio cholerae* O1 Results in Innate Resistance to Ciprofloxacin▽

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A mini-Tn*5* insertion into a ciprofloxacin (CIP)-resistant mutant of *Vibrio cholerae* O1 revealed that overexpression of the *vca0421* gene, which encodes a hypothetical protein, in the CIP-resistant mutant carrying a mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene causes sensitization to CIP. We propose a new intrinsic mechanism of resistance to fluoroquinolones due to the inherently reduced expression of the *vca0421* gene in *V. cholerae* O1.

Fluoroquinolones, which display potent antibacterial activity against *Vibrio cholerae* O1 and O139, have been used in the clinical treatment of cholera (13). However, increased therapeutic use of fluoroquinolones has resulted in the appearance of fluoroquinolone-resistant strains of *V. cholerae* O1 and O139 in clinical isolates from around the world (3, 9, 10).

In addition to *V. cholerae* O1 and O139, many other bacterial species have developed clinical resistance to fluoroquinolones. The molecular basis of this antibiotic resistance has been studied extensively (2). Most of the acquired resistance can be attributed to mutations in the genes encoding DNA gyrase or topoisomerase IV (Topo IV). Bacterial resistance to fluoroquinolones can also be conferred by increased expression of multidrug efflux pumps or reduced expression of outer membrane proteins, such as porins, resulting in reduced intracellular concentrations of antibiotics (5). DNA gyrase consists of GyrA and GyrB subunits, encoded by the *gyrA* and *gyrB* genes, respectively (1, 12). Topo IV is composed of ParC and ParE subunits, encoded by the *parC* and *parE* genes, respectively, the amino acid sequences of which are homologous to some degree with those of GyrA and GyrB, respectively (4, 11). The majority of the quinolone resistance mutations have been shown to map to a relatively small region at the N terminus of GyrA, corresponding to the 67th through the 106th amino acid residues in *Escherichia coli* K-12; this region is called the quinolone resistance-determining region (QRDR) (5, 14). Quinolone resistance mutations in the *parC* genes were also detected in the region corresponding to the QRDR of ParC (5).

In this study, we attempted to reveal a novel mechanism of *V. cholerae* O1 resistance to fluoroquinolones. Such data will be key to developing new antibiotics that are effective against fluoroquinolone-resistant *V. cholerae* O1 strains.

Isolation of ciprofloxacin (CIP)-resistant mutants of 569B was carried out as follows. A single colony of strain 569B was inoculated into 5 ml of Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, MI), and 0.2-ml aliquots of the overnight culture were plated onto a Mueller-Hinton agar (MHA; Difco Laboratories, Detroit, MI) plate containing 0.008 μg of CIP per ml. A resistant colony (the first-step mutant) was selected. The emerging clone was purified on an MHA plate without the antimicrobial agent. The second-step (selective concentration of ciprofloxacin, 0.015 μg/ml) and third-step (selective concentration of ciprofloxacin, 0.25 μg/ml) mutants were obtained in the same manner from the first- and second-step mutants, respectively. A CIP-resistant mutant that grew on the selective medium containing CIP at 0.25 μg/ml was chosen (designated CIP0.25-1), and the MIC of CIP against the CIP0.25-1 strain was determined by the 2-fold agar dilution method recommended by the Japan Society of Chemotherapy (http://www.chemotherapy.or.jp/index.html). The MIC of CIP against the CIP0.25-1 strain was 250-fold higher than that against the wild-type strain (Table 1). As the CIP0.25-1 strain showed a high level of resistance to CIP, we investigated whether point mutations in the QRDRs of the *gyrA* and *parC* genes in the CIP-resistant mutant could be detected. Amplification of QRDRs was carried out by using 5′-VC-*gyrA* (5′-AATGTGCTGGGCAACGACTGG-3′) and 3′-VC-*gyrA* (5′-GTGCGCGATTTTCGACATACG-3′) primers for the *gyrA* gene and by using 5′VC-*parC* (5′-GTCTGAGTTGGGTCTCTCGGC-3′) and 3′-VC-*parC* (5′-AGAATCTCGGCAAACTTTGACAG-3′) primers for the *parC* gene, as described previously (2). Sequences of the amplified *gyrA* and *parC* QRDRs were determined by using an ABI Prism TM310 genetic analyzer (Applied Biosystems, Foster City, CA). A single point mutation in the QRDR of the *gyrA* gene (G-to-T substitution at nucleotide position 248; i.e., Ser-to-Ile substitution at deduced amino acid position 83) was detected in the CIP0.25-1 strain compared with the wild-type strain. However, there was no mutation in the QRDR of the *parC* gene in this strain (Table 2). Because only one point mutation in the QRDR of the *gyrA* gene was detected in the CIP0.25-1 strain, which exhibits a high level of resistance to CIP, other genetic mechanisms contributing to this high-level resistance were likely to be present.

To reveal an unknown resistance mechanism in the CIP0.25-1 strain, we carried out a mini-Tn*5* insertion to obtain a CIP-susceptible revertant (designated mTn-321). The mini-Tn*5* was integrated into the chromosome of the CIP0.25-1 strain as follows. pUTmini-Tn*5*Sm/Sp was transformed into *E.*

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TABLE 1. Antibacterial activities of fluoroquinolones against *V. cholerae* 569B, CIP0.25-1, and mTn-321

Agent	MIC (μg/ml)		
	569B	CIP0.25-1	mTn-321
Ciprofloxacin	0.004	1	0.0625
Norfloxacin	0.0078	2	0.125
Sparfloxacin	0.004	0.125	0.0625
Enoxacin	0.0313	2	0.25
Tetracycline	0.5	4	0.5

TABLE 3. Antibacterial activities of fluoroquinolones against *E. coli* JM109(ptac85) and JM109(ptac85-vca0421)

Agent	MIC (μg/ml)	
	JM109(ptac85)	JM109(ptac85-vca0421)
Ciprofloxacin	0.5	0.25
Norfloxacin	1	1
Sparfloxacin	0.25	0.125
Enoxacin	4	2
Chloramphenicol	16	16
Tetracycline	16	8

coli SM10λpir and then mobilized from the resulting strain into CIP0.25-1 by conjugation on a membrane filter as described previously (8). The transconjugants were selected on TCBS (thiosulfate-citrate-bile-sucrose) agar (Difco Laboratories) supplemented with spectinomycin (25 μg/ml). To confirm that the transconjugants resulted from the transposition of the mini-Tn*5* module, PCR was carried out to detect the 2.1-kb fragment of the mini-Tn*5* sequence by using mTn5-I (5′-CGGTGATTGATTGAGCAAGC-3′) and mTn5-O (5′-CTGACTCTTATACACAAGTTCG-3′) primers. A transconjugant (mTn-321), of which the MIC against CIP was 16-fold lower than that of CIP0.25-1 (Table 1), was selected out of the transconjugants which showed possession of the mini-Tn*5* module. From mTn-321, a Sau3AI fragment containing the mini-Tn*5* module was cloned into pUC119, and the upstream and downstream sequences flanking the mini-Tn*5* module were then determined. These sequences were subjected to a BLAST search against the *V. cholerae* O1 genome released by The Institute for Genomic Research at http://www.tigr.org. The BLAST search revealed that the mini-Tn*5* was inserted 110 bp upstream of the *vca0421* gene (GenBank accession no. AE003853), which encodes a hypothetical protein (GenBank accession no. AAF96327). Furthermore, no obvious open reading frame (ORF) was detected in the region upstream of the *vca0421* gene. MICs of fluoroquinolones and tetracycline (TC) against the wild-type strain, CIP0.25-1, and mTn-321 were determined by the 2-fold agar dilution method (Table 1). CIP0.25-1 showed a high level of resistance to all fluoroquinolones tested. In contrast, the mTn-321 revertant was susceptible to all fluoroquinolones tested.

To examine the relationship between *vca0421* gene expression and CIP susceptibility, the *vca0421* gene was engineered for overexpression in *E. coli* JM109. Specifically, the effect of fluoroquinolones and TC on the growth of *E. coli* JM109 harboring either the ptac85-vca0421 plasmid or vector alone was tested. As shown in Table 3, overexpression of the ptac85-vca0421 plasmid resulted in sensitization of *E. coli* against fluoroquinolones and tetracycline. Furthermore, overexpression experiments were performed in *V. cholerae* O1 569B and CIP0.25-1. Semiquantitative reverse transcription-PCR (RT-PCR) did not detect expression of the *vca0421* gene under regulation of the *tac* promoter or the promoter of the cholera toxin gene (*ctx*) (GenBank accession no. X58785.1) from *V. cholerae* O1 on the broad-host-range vector pCVD503 (6) in either strain (data not shown). Amplification of a region including the *vca0421* gene and its native promoter was also unsuccessful, maybe because of the secondary-structure formation of the promoter region (data not shown).

TABLE 2. Mutations detected in the *gyrA* and *parC* QRDR sequences of the 569B, CIP0.25-1, and mTn-321 strains

Strain	*gyrA* QRDR sequence		*parC* QRDR sequence	
	Residue or base change	Residue or amino acid change	Residues or base change	Residues or amino acid change
569B	G at 248	Ser at 83	nt 162 to 411	aa 54 to 137
CIP0.25-1	G → T at 248	Ser → Ile at 83	No change	No change
mTn-321	G → T at 248	Ser → Ile at 83	No change	No change

Then, expression levels of the *vca0421* gene in the three *V. cholerae* O1 strains (wild-type strain, CIP-resistant mutant, and mTn-321 revertant) were compared by semiquantitative RT-PCR as described previously (7). For RT-PCR, bacterial cells were cultured in LB (Difco Laboratories) at 25°C overnight. Total RNAs from the three strains were isolated with Isogen (Nippongene, Tokyo, Japan) by using the method recommended by the manufacturer. The RNAs were purified again after treatment with RNase-free DNase I (Takara Bio, Otsu, Japan), and the amount and purity of the RNAs were determined by measuring the A_{260}/A_{280} ratio. A total of 100 ng of the RNA was used to amplify the *vca0421* and *gyrB* transcripts by RT-PCR. The *gyrB* transcript level was used as the internal control for RT-PCR as described previously (7). RT-PCR was performed using a SuperScript one-step RT-PCR with Platinum *Taq* system (Invitrogen, Carlsbad, CA) and primers for *vca0421* (5′-VC-*vca0421* [5′-GAGAGGATCCATGAAAAAATTGTTAATGGTACTG-3′] and 3′-VC-*vca0421* [5′-AGAGGTCGACTTACATAACGCACTCTTTCG-3′]) and *gyrB* (5′-VC-*gyrB* [5′-ATGTCGAACAATTACGATTCATC-3′] and 3′-VC-*gyrB* [5′-CAGTACAGTCATGATGACTTCTG-3′]). The RT-PCR was performed with the following cycle profile: 35 cycles of *vca0421* gene annealing at 48°C and 35 cycles of *gyrB* annealing at 50°C. The RT-PCR products (446 bp for *vca0421* and 294 bp for *gyrB*) were subjected to agarose gel electrophoresis (2% gel) and visualized by staining with ethidium bromide. Semiquantitative RT-PCR was carried out as described previously (7), with slight modifications. The relative expression level of the *vca0421* transcript was measured by normalizing the PCR product of the *vca0421* gene to that of the *gyrB* gene. After the densitometric intensities of the RT-PCR products were quantified with NIH Image software, the relative expression level of the *vca0421* transcript was calculated as the ratio of the final RT-PCR product of the *vca0421* gene to that of the *gyrB* gene. The amount of contaminating chromosomal DNA in each sample was determined in control reactions without reverse transcriptase. Consequently, it was

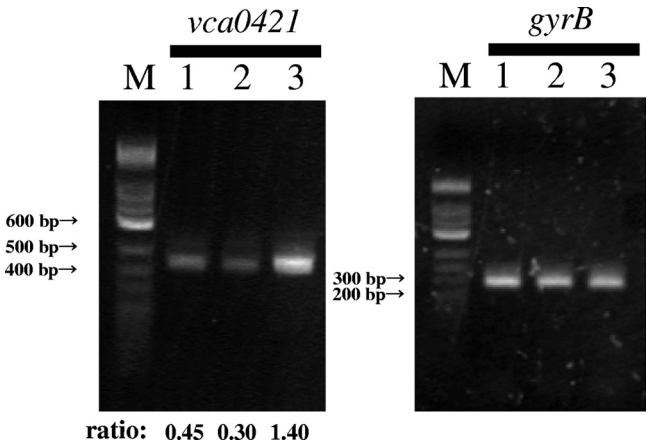

FIG. 1. RT-PCR analysis of the *vca0421* transcript levels. Lane M, 100-bp DNA ladder marker; lane 1, wild-type strain; lane 2, CIP-resistant mutant CIP0.25-1; lane 3, mTn-321. The *gyrB* gene was used as an internal control. The relative expression level of the *vca0421* transcript was calculated as the ratio of the RT-PCR product of the *vca0421* gene to that of the *gyrB* gene (values shown at bottom).

found that the relative expression level of the *vca0421* gene in the mTn-321 revertant was 4.7-fold higher than that in the CIP-resistant mutant (Fig. 1). However, in the mTn-321 revertant, there was no change in the single point mutation in the QRDR of the *gyrA* gene that was detected in the CIP0.25-1 strain (Table 2). In addition, we performed sequence analysis of the 250-bp upstream regions of the *vca0421* ORF in the wild-type and CIP0.25-1 strains and found that there was no difference between the two sequences.

Taken together, our results suggest that overexpression of the *vca0421* gene in a CIP-resistant mutant carrying a mutation in the QRDR of the *gyrA* gene causes sensitization to CIP. However, the precise mechanism underlying this observed sensitization remains unclear. In conclusion, we propose a new intrinsic mechanism of *V. cholerae* O1 resistance to fluoroquinolones due to the inherently reduced expression of the *vca0421* gene.

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