

Cloning of a Novel Gene for Quinolone Resistance from a Transferable Plasmid in *Shigella flexneri* 2b

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A novel gene for quinolone resistance was cloned from a transferable plasmid carried by a clinical isolate of *Shigella flexneri* 2b that was resistant to fluoroquinolones. The plasmid conferred low-level resistance to quinolones on *Escherichia coli* HB101. The protein encoded by the gene showed 59% amino acid identity with Qnr.

Quinolone resistance is usually caused by mutations in the chromosomal genes for DNA gyrase and DNA topoisomerase IV, the targets of quinolones (1, 3). Plasmid-mediated resistance was first discovered in a clinical strain of *Klebsiella pneumoniae* isolated in Alabama (5). This plasmid, pMG252, was a multiresistance-encoding plasmid and contained the gene for quinolone resistance, *qnr*. The *qnr* gene has been found to encode a 218-amino-acid protein, and the purified Qnr protein has been shown to protect DNA gyrase activity in vitro (8). The *qnr* gene has since been detected in more than 20 clinical strains of *K. pneumoniae* and *E. coli* isolated in the United States and China (4, 7, 10, 11).

At the beginning of October 2003, an outbreak of food poisoning caused by *Shigella flexneri* 2b occurred in Aichi Prefecture, Japan. Eight clinical strains isolated from the patients were subjected to pulsed-field gel electrophoresis analysis for epidemiological investigation. They were judged to be clones from the same origin on the basis of the pulsed-field gel electrophoresis pattern obtained. We also determined the drug sensitivities of these isolates. Seven of the eight clones had the same pattern of drug resistance. However, one had additional resistance to fluoroquinolones and cephaloridine. In the course of investigating the mechanism of acquisition of the additional drug resistance, we found a plasmid carrying resistance genes and cloned a novel gene responsible for quinolone resistance. This is the first report of a gene for quinolone resistance on the conjugative plasmid in *Shigella*.

Strains and drug resistance. Nine strains of *S. flexneri* 2b were isolated from eight patients. Strains 1 to 8 were isolated from different patients from 1 to 9 October 2003. Strains 8 and 8' were from the same patient and were isolated on 8 and 20 October, respectively. This patient was treated with levofloxacin.

With antibiotic disks (KB disk; Eiken Chemical Co., Tokyo, Japan), patterns of resistance to 12 drugs were examined. Patterns of seven isolates of *S. flexneri* 2b were the same. They were

resistant to ampicillin, streptomycin, kanamycin, chloramphenicol (CHL), tetracycline, and nalidixic acid (NAL). However, strain 8 (and strain 8') was resistant to three additional drugs, norfloxacin, ciprofloxacin, and cephaloridine (CER). All isolates were sensitive to cefotaxime, fosfomicin, and trimethoprim-sulfamethoxazole.

The mutations in the quinolone resistance-determining region (12) of the *gyrA* and *parC* genes were examined. The quinolone resistance-determining region cloned by PCR (9) was sequenced by the IR dye terminator method with the LI-COR 4200 IR² system (LI-COR, Lincoln, Nebr.). The following mutations were found in all of the isolates: Ser-83 to Leu in the *gyrA* gene and Ser-80 to Ile in the *parC* gene.

Plasmid profiles and conjugation experiments. Plasmid profiles revealed that strain 8 (and strain 8') had a unique plasmid of about 47 kb that was not present in the other seven isolates. The plasmid was designated pAH0376.

A conjugation experiment was carried out with *E. coli* HB101 (F⁻ *hdsS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20* [Str^r] *xyl-5 mtl-1 supE44 leuB6 thi-1*) as the recipient, with selection for pAH0376-encoded cephaloridine resistance. *Shigella* strain 8 was the donor. Overnight cultures of *Shigella* cells (10 μ l) and HB101 cells (1 ml) were added to fresh Luria-Bertani (LB) broth (5 ml) and incubated for 6 h. Transconjugants were selected on Sorbitol-MacConkey agar (Eiken Chemical Co.) plates containing cephaloridine at 5 μ g/ml. HB101 cells generate red colonies on Sorbitol-MacConkey plates (Sorbitol fermented), whereas *Shigella* cells generate pinkish colonies (not Sorbitol fermented). Transconjugant HB101 appeared as red colonies on the plates. pAH0376 was transferred to HB101 (Fig. 1, lane HB101-T). Transconjugant HB101 showed low resistance to nalidixic acid (MIC, 16 μ g/ml), ciprofloxacin (MIC, 0.25 μ g/ml), and cephaloridine (MIC, 16 μ g/ml) and in addition high resistance to ampicillin (MIC, 512 μ g/ml). The transconjugant was sensitive to other drugs except streptomycin, to which HB101 is resistant.

A second conjugation experiment was performed to transfer pAH0376 from transconjugant HB101 to the *Shigella* strains. Transconjugants were selected on *Salmonella-Shigella* agar (Eiken Chemical Co.) plates containing cephaloridine at 5 μ g/ml. Growth of *E. coli* is suppressed on *Salmonella-Shigella* agar plates. Two *S. flexneri* 2b strains (5 and 7) were the

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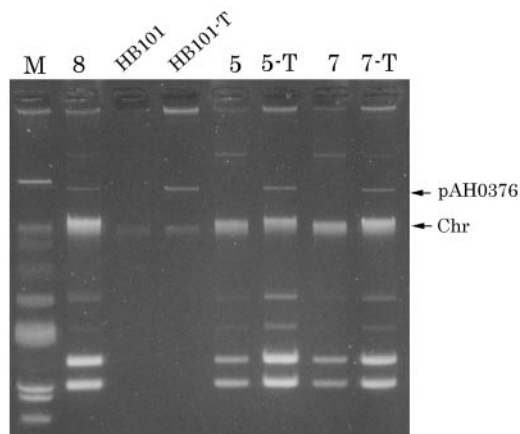


FIG. 1. Transmission of plasmid pAH0376 to *E. coli* HB101 and outbreak strains of *S. flexneri* 2b. Lanes: M, molecular size marker strain V517; 8, strain 8; HB101-T, transconjugant of HB101 with pAH0376 transmitted from strain 8; 5 and 7, strains 5 and 7; 5-T and 7-T, transconjugants of strains 5 and 7 with pAH0376 transmitted from HB101-T. The positions of plasmid pAH0376 and chromosomal DNA (Chr) are indicated on the right.

recipients. The recipient strains were sensitive to ciprofloxacin (MIC, 1 µg/ml), cephaloridine (MIC, 4 µg/ml), and ampicillin (MIC, 128 µg/ml). Plasmid pAH0376 was transferred to these *Shigella* strains, and the transconjugants showed the same level of drug resistance as strain 8: the MIC of ciprofloxacin was 8 µg/ml, that of cephaloridine was 16 µg/ml, and that of ampicillin was 512 µg/ml (Fig. 1). The results indicated that the difference in the drug resistance patterns observed in the outbreak strains of *S. flexneri* 2b was caused by conjugative plasmid pAH0376.

Cloning of resistance-encoding genes. pAH0376 was isolated from transconjugant HB101 and digested with EcoRI or HindIII. The resulting fragments were cloned into vector pBC SK (+) (Stratagene, La Jolla, Calif.), and recombinants were transformed into HB101. Transformants were selected on LB broth plates containing CHL (30 µg/ml; the selection drug for the pBC vector) and NAL (4 µg/ml) or CHL and cephaloridine (CER; 5 µg/ml). A clone carrying a plasmid with a 2.6-kb HindIII insert was isolated on the NAL-CHL plates. This clone (pBC-H2.6) showed the same level of resistance to quinolones as pAH0376. The nucleotide sequence of the 2,642-bp insert was determined. We identified an open reading frame (ORF) of 657 bp encoding a 218-amino-acid polypeptide. The PCR fragment of the 839 bp encompassing the entire transcription unit of the ORF (-96 from ATG to +86 from TAA) was amplified with pAH0376 as the template. The PCR product was cloned into a pGEM-T vector (Promega, Madison, Wis.) and transformed into JM109 (Takara Bio, Otsu, Japan). The resultant construct (pGEM-ORF) was isolated, its nucleotide sequence was verified, and it was transformed into HB101. The transformant was isolated on broth plates containing ampicillin at 50 µg/ml (the selection drug for the pGEM vector). pGEM-ORF conferred the same level of quinolone resistance as pAH0376, indicating that this ORF was responsible for quinolone resistance.

A BLAST analysis of deduced amino acid sequences revealed that the gene product exhibited significant homology to the Qnr protein, with 59% amino acid identity. Besides Qnr, two other proteins also revealed high similarity. Hypothetical proteins of *Photobacterium profundum* (CAG22829) and *Vibrio vulnificus* (AAO07889) showed 64 and 53% amino acid identity, respectively. An alignment of the ORF-encoded protein, Qnr, and the hypothetical proteins of *P. profundum* and *V.*

QnrS	1:METYNHTYRHHNFSHKDLSDLTFTACTFIRSDFRANLRDITFVNCKFIEQGDIEGCHF	60
Qnr	1:MDI IDKVFQQEDFSRQDLSDSRFRRCRFYQCDFSHCQLQDASFEDCSFIESGAVEGCHFS	60
CAG22829	1:MAKDYEVEFNQVDFSNQDLSDSYFKGCKFFRCNFRNTLRDAKFDDCVFIEQGAIEGCHF	60
AAO07889	1:MKYQGQHFHREDFSNQDLQQAIFDHCHFIECNFDRADLTDSQFIHCRFIEASSIEGCSFR	60
QnrS	61:VADLRDASFQCCQLAMANFNSNANCYGIEFRACDLKGANFSRTNFAHQVSNRMFYCSAFIS	120
Qnr	61:YADLRDASFKACRLSLANFSGANCFGIEFRECDLKGANFSRARFYNQVSHKMYFCSAYIS	120
CAG22829	61:YSDLRDSSEFKNCKLSLSNFKGAKCFGIEFRSCLKGANFIQASFSNQISNSMYFCSAYIT	120
AAO07889	61:YTKLREASFHKCMMAMAQFTGADCFGIEFRECDLKGSNFSQANFSNRISHKAYFCSAYIT	120
QnrS	121:GCNLSYANMERVCLEKCELFENRWIGTNLAGASLKESDLRSGVFSSEVWVGQFSLQGANLC	180
Qnr	121:GCNLAYTNLSGQCLEKCELFENNSNANLSGASLMGSDLRSGTFSRDCWQVNLRGCDLT	180
CAG22829	121:GCNLSYANFERQCIKCDLFENRWIGANLQGASLKGSDLRSGSFSSEDCWQFYIQDCDLS	180
AAO07889	121:GCNLNYSNFERAMLEKCDLFENRWGANLSGASLKGSDLRSGEFSPEQWGSFIVEECDLT	180
QnrS	181:HAELDGLDPRKVDTSKGIKIAAWQQLILEALGIVVYPD	218
Qnr	181:FADLDGLDPRRVNLEGVKICAWQQLLEPLGIVLVPD	218
CAG22829	181:NSELNGLNPRKVDLSGVKICVWQQLLEQLGLIVLPN	218
AAO07889	181:HIELDGLDVRVSLHGAKICDWQQAQLLESFGLIVVPG	218

FIG. 2. Alignment of deduced amino acid sequences of the ORF-encoded protein examined in this study (QnrS), Qnr, and hypothetical proteins of *P. profundum* (CAG22829) and *V. vulnificus* (AAO07889). Identical amino acids are highlighted.

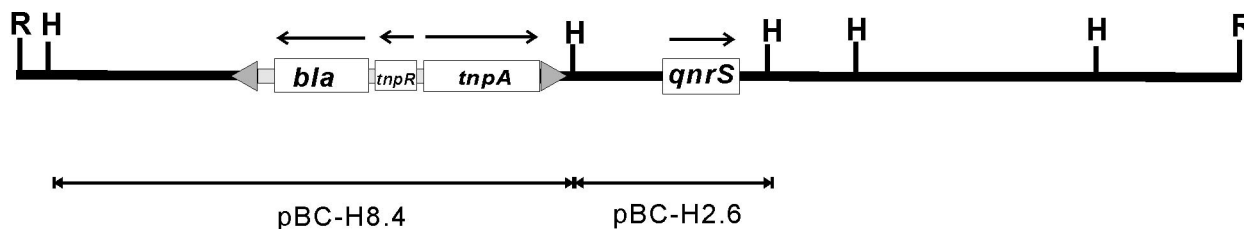


FIG. 3. Map of the EcoRI fragment of pAH0376. The *qnrS* gene and the TEM-1 β -lactamase gene are indicated. The other two open squares are the genes for resolvase (*tnpR*) and transposase (*tnpA*), respectively. The triangles represent 38-bp inverted repeats on both sides of the Tn3-like transposon. H, HindIII; R, EcoRI.

vulnificus is shown in Fig. 2. The four proteins all consisted of 218 amino acids and had 43% of their amino acids in common. In view of the high similarity to the Qnr protein, it is conceivable that the product of the new gene is a homologue of Qnr. We designated the new gene *qnrS* and its product QnrS. The resistance-encoding activity of *qnrS* was similar to that of *qnr*. Both conferred a low level of quinolone resistance and had an additive effect on quinolone resistance due to defined DNA gyrase alterations (6).

A recombinant clone carrying a plasmid with an ~20-kb EcoRI insert (pBC-R20) was isolated on both NAL-CHL and CER-CHL plates, and a clone with an 8.4-kb HindIII insert (pBC-H8.4) was isolated on CER-CHL plates. A restriction map of pBC-R20 revealed that the 2.6- and 8.4-kb HindIII fragments were involved in the EcoRI insert (Fig. 3). The nucleotide sequence analysis identified a Tn3-like sequence containing a TEM-1 β -lactamase gene (2). There was no element for an integron in the region sequenced. Further investigations are required to resolve how the *qnrS* gene was acquired.

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the EMBL/GenBank/DDBJ databases and assigned accession number AB187515.

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