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Original article

Characterization of *Campylobacter jejuni* DNA gyrase as the target of quinolones

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ABSTRACT

Quinolones have long been used as the first-line treatment for *Campylobacter* infections. However, an increased resistance to quinolones has raised public health concerns. The development of new quinolone-based antibiotics with high activity is critical for effective, as DNA gyrase, the target of quinolones, is an essential enzyme for bacterial growth in several mechanisms. The evaluation of antibiotic activity against *Campylobacter jejuni* largely relies on drug susceptibility tests, which require at least 2 days to produce results. Thus, an *in vitro* method for studying the activity of quinolones against the *C. jejuni* DNA gyrase is preferred. To identify potent quinolones, we investigated the interaction of *C. jejuni* DNA gyrase with a number of quinolones using recombinant subunits. The combination of purified subunits exhibited DNA supercoiling activity in an ATP dependent manner. Drug concentrations that inhibit DNA supercoiling by 50% (IC_{50}s) of 10 different quinolones were estimated to range from 0.4 (sitafloxacin) to >100 μg/mL (nalidixic acid). Sitafloxacin showed the highest inhibitory activity, and the analysis of the quinolone structure-activity relationship demonstrated that a fluorine atom at R-6 might play the important role in the inhibitory activity against *C. jejuni* gyrase. Measured quinolone IC_{50}s correlated well with minimum inhibitory concentrations ($R = 0.9943$). These suggest that the *in vitro* supercoiling inhibition assay on purified recombinant *C. jejuni* DNA gyrase is a useful and predictive technique to monitor the antibacterial potency of quinolones. And furthermore, these data suggested that sitafloxacin might be a good candidate for clinical trials on campylobacteriosis.

1. Introduction

Campylobacter jejuni infection is one of the most commonly identified bacterial causes of gastroenteritis worldwide, and it occurs more frequently than infections caused by other enteric pathogens. *Campylobacter* infection causes diarrhea in approximately 400–500 million people globally each year [1,2]. Most patients with *Campylobacter* infection develop a self-controlled condition that does not require antibiotics. Nevertheless, an antibiotic treatment is recommended when the *Campylobacter* infection is severe or affects an immunocompromised host [3]. Quinolones are a family of antimicrobials for the treatment of *Campylobacter* infections in human and animals [1,2]. However, the introduction of quinolones in veterinary medicine has been reported to cause an emergence of quinolone-resistant

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Campylobacter [4–6]. In fact, quinolone resistance in *Campylobacter* from food animals is now recognized as an emerging public health problem [3,7]. Testing for quinolone susceptibility is essential to provide guidance to physicians and veterinarians on the appropriate treatment for *Campylobacter* infections. *C. jejuni* is a slow-growing bacterium that requires microaerophilic conditions and supplemented growth media. Variations in the culture media and the incubation conditions, namely, atmosphere, temperature, and time of incubation, could affect the results of antimicrobial susceptibility tests. Therefore, it is highly desirable to develop a simple and rapid test for quinolone susceptibility in *Campylobacter*.

Quinolones belong to a family of broad-spectrum synthetic antimicrobials. In bacteria, the target of quinolones are the essential enzymes DNA gyrase and DNA topoisomerase IV, belonging to the bacterial type II topoisomerase. DNA gyrase is unique in that it catalyzes the negative supercoiling of DNA and is essential for DNA replication, transcription and recombination [8]. On the contrary, topoisomerase IV has a specialized role in chromosome segregation. Complete genome sequencing of *C. jejuni* revealed the lack of genes encoding topoisomerase IV [9–11] and thus DNA gyrase turned out to be the sole target of quinolones in *Campylobacters*. Quinolone inhibit DNA supercoiling by stabilizing the complex between gyrase and the cleaved DNA, interrupting the propagation of the replication fork. When the DNA gyrase cleaves the DNA, the antibiotics prevent relegation of the broken strand, resulting in a quinolone-enzyme-DNA complex that leads to inhibition of DNA replication [12,13]. However, because of increased quinolone resistance in *C. jejuni*, an *in vitro* method that would accelerate the identification of more potent quinolones against *C. jejuni* is needed for its treatment and effective control.

The DNA gyrase consists of two subunits A (GyrA) and B (GyrB) encoded by *gyr*A and *gyr*B, respectively. In order to measure inhibitory activity against *C. jejuni*, we reconstituted DNA gyrase *in vitro*, expressed both GyrA and GyrB in *Escherichia coli*, and used their purified forms to evaluate a group of 10 quinolones.

2. Materials and methods

2.1. Reagents

Ciprofloxacin (CIP), gatifloxacin (GAT), levofloxacin (LVX), sparfloxacin (SPX), enrofloxacin (ENR) and ofloxacin (OFX) were purchased from LKT Laboratories, Inc. (St. Paul, MN). Oxolinic acid (OXO) and nalidixic acid (NAL) were purchased from Wako Pure Chemicals Ltd. (Tokyo, Japan). Moxifloxacin (MXF) was obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Sitafloxacin (SIT) was a gift from Daiichi Sankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). Oligonucleotide primers were synthesized by Life Technologies (Carlsbad, CA). A Ni-nitrolotriacetic acid (Ni-NTA) protein purification kit was purchased from Life Technologies. Restriction enzymes were obtained from New England BioLabs, Inc. (Ipswich, MA). Supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom). A protease inhibitor cocktail (Complete Mini, EDTA free) was purchased from Roche Applied Science (Mannheim, Germany).

2.2. Bacterial strains and plasmids

E. coli strain TOP-10 (Life Technologies Corp., Carlsbad, CA) was used as the cloning host. The pUC118-*Hin*cII/BAP plasmid (Takara Bio, Kyoto, Japan) was used to clone amplified DNA fragments. *E. coli* strains BL-21 (DE3)/pLysS (Merck KGaA, Darmstadt, Germany) were used for protein expression. Vector plasmid pET-20b (+) (Merck KGaA) was used to construct expression plasmids for *C. jejuni* proteins GyrA and GyrB.

2.3. Quinolones susceptibility testing

C. jejuni ATCC33560 was grown on Mueller-Hinton agar (MHA; Oxoid Ltd., Basingstoke, UK), and the minimum inhibitory concentrations (MICs) for this strain were determined by a broth dilution method. Briefly, 1 μl of suspension was inoculated into Mueller-Hinton broth (MHB; Oxoid Ltd.) supplemented with 5% defibrinated sheep blood and containing 2-fold serial dilutions of the quinolone. The microdilution tray was incubated at 37 °C under microaerophilic conditions (10% CO_2, 5% O_2, and 85% N_2) for 48 h. The MIC was predetermined as the lowest concentration of quinolone to cause a complete growth inhibition.

2.4. Construction of DNA gyrase expression vectors

DNA fragments encoding *gyr*A and *gyr*B were amplified by polymerase chain reaction using the primers listed in Table 1. The reaction mixture (25 μl) consisted of primeSTAR GXL buffer (Mg^{2+} plus); 200 μM each of dATP, dCTP, dGTP, and dTTP; 10 ng DNA from *C. jejuni* ATCC33560; 2.0 units of PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Kyoto, Japan); and 0.4 μM of each primer. PCR was carried out in an iCycle Thermal Cycler (Bio-Rad Laboratories GmbH, California, US) under the following amplification conditions: pre-denaturation at 98 °C for 2 min, 35 cycles of denaturation at 98 °C for 10 s, annealing 50 °C for 10 s, extension 72 °C for 3 min, and a final extension step at 72 °C for 2 min. The PCR products corresponding to the 2.5-kb *gyr*A and 2.3-kb *gyr*B fragments were ligated into the pUC118-*Hin*cII/BAP plasmid and transformed into *E. coli* Top10 competent cells, according to the manufacturer's instructions. Recombinant plasmids were recovered from white colonies and digested with *Nde* I and *Xho* I, and the obtained DNA fragments were ligated into *Nde* I-*Xho* I-digested pET-20b and transformed into *E. coli* Top10. Recombinant clones were selected from the resistant colonies on Luria–Bertani (LB) agar containing ampicillin (100 μg/mL). The nucleotide sequences of the DNA gyrase genes in the plasmids were analyzed using the ABI Prism BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Cycle sequencing products were subsequently analyzed in an ABI PRISM 3130x automated genetic analyzer (Applied Biosystems). The sequences were compared with their respective wild-type sequences using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.5. Recombinant expression and purification of DNA gyrase

DNA gyrase subunits were expressed and purified as previously described [14–16], with modifications. Briefly, expression vectors carrying *C. jejuni gyr*A and *gyr*B were transformed to *E. coli* BL 21(DE3)/pLysS. The transformants were grown in LB medium in the presence of 100 μg/mL ampicillin to the log phase. Expression of GyrA and GyrB was induced with the addition of 1 mM isopropyl β-D-thiogalactopyranoside (Wako Pure Chemicals Industries Ltd.), and further incubation was conducted for 16 h at 18 °C. Harvested *E. coli* was lysed by sonication at 30% duty cycle, 10 cycles of 40 s on and 40 s off using Sonifier 250 (Branson, Danbury, CT). After supernatants were centrifuged (10,000 × *g*) for 30 min, recombinant DNA gyrase subunits were purified by Ni-NTA resin column chromatography and dialyzed against DNA gyrase dilution buffer (50 mM Tris–HCl pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA). Protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Wako Pure Chemicals Industries Ltd.) with a protein molecular weight marker (New England Biolab).

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