Opposite effects of cefoperazone and ceftazidime on S-ribosylhomocysteine lyase/autoinducer-2 quorum sensing and biofilm formation by an Escherichia coli clinical isolate

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Abstract. To investigate the effects of subminimum inhibitory concentrations of cephalosporins on bacterial biofilm formation, the biofilm production of 52 Escherichia (E.) coli strains was examined following treatment with cephalosporin compounds at 1/4 minimum inhibitory concentrations (MICs). Ceftazidime (CAZ) inhibited biofilm formation in seven isolates, while cefoperazone (CFP) enhanced biofilm formation in 18 isolates. Biofilm formation of E. coli E42 was inhibited by CAZ and induced by CFP. Therefore, using reverse transcription-polymerase chain reaction, the expression of the luxS gene in E. coli, CAZ at 1/4 MIC reduced luxS mRNA levels and the production of AI-2, whereas CFP at 1/4 MIC had the opposite effect. AS-ODNs targeting luxS significantly decreased the aforementioned inhibitory effects of CAZ and the induction effects of CFP on E. coli biofilm formation. Therefore, biofilm formation by the E. coli clinical isolate E42 was evoked by CFP but attenuated by CAZ at sub-MICs, via a luxS/AI-2-based quorum sensing system.

Introduction

Bacterial biofilms are a polymeric matrix produced by the bacterial cells embedded within it. The biofilm and cells together constitute a structured community that is a common cause of numerous persistent infections (1). Furthermore, such a community enables certain bacteria to control their individual gene expression via quorum sensing (QS), by detecting autoinducers secreted by themselves or their neighbors (2).

Protected within the biofilm, the bacteria are more resistant to antibiotics than their planktonic forms (3). An important example is Escherichia (E.) coli, a commonly encountered pathogen that is often responsible for community- and hospital-acquired infections. Isolates of E. coli that are harbored within the biofilm are often resistant to antibiotic treatment (4).

Emerging evidence strongly suggests that antibiotics at sub-minimum inhibitory concentrations (MICs) may nonetheless interfere with bacterial functions. These effects may have clinical relevance as bacteria are commonly exposed to sub-MICs of antibiotics at a certain period, particularly at the beginning and end of the treatment (5).

Third-generation cephalosporins, a class of β-lactam antibiotics, are widely used in the treatment of bacterial infections caused by gram-negative bacteria, such as E. coli. Examples of cephalosporins include ceftazidime (CAZ), cefoperazone (CFP), ceftriaxone and cefotaxime. Several studies have demonstrated that CAZ at sub-MICs inhibited QS in Pseudomonas aeruginosa and Burkholderia pseudomallei, thereby decreasing the synthesis of a range of QS-regulated virulence factors (6,7).

The present study aimed to investigate the effects that sub-MICs of cephalosporins may have on the biofilm production of E. coli isolates. To investigate these effects, the biofilm production of 52 E. coli reference strains and clinical isolates following treatment with 1/4 MICs of third-generation cephalosporins were observed. In one E. coli clinical isolate, CFP and CAZ exerted opposite effects on biofilm formation. The mechanisms of these effects were then examined in that isolate.

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Materials and methods

**Bacterial strains and growth conditions.** In order to investigate the effects of sub-MICs of third-generation cephalosporins on the biofilm formation of *E. coli*, at the beginning of the study each of the three *E. coli* reference strains (ATCC700926, ATCC35218 and DH5α) and 49 clinical isolates were treated separately with four third-generation cephalosporins [Ceftazidime (CAZ; Sigma-Aldrich, Shanghai, China), ceftriaxone, cefotaxime or cefoperazone (CFP) (all from National Institutes for Food and Drug Control, Beijing, China)] at 1/4 MICs. The study was approved by the Ethics Committee of Southwest Hospital, Third Military Medical University, (Chongqing, China).

The ATCC700926, ATCC35218, DH5α and BAA1117 (*Vibrio harveyi* BB170) strains were purchased from the American Type Culture Collection (Manassas, VA, USA). A total of 49 *E. coli* isolates collected from Southwest Hospital (Chongqing, China) between January 2009 and February 2009 were used in this study. Written informed consent from the patient/patient's family was obtained prior to the study.

Among the *E. coli* examined, E42, isolated from the pus of a surgical patient who had undergone a curative resection of a colorectal carcinoma, was noteworthy as formation of its biofilm was suppressed by 1/4 MIC CAZ, while it was enhanced by 1/4 MIC CFP. To examine the underlying mechanisms controlling these opposite effects, E42 was selected for further investigation in the subsequent experiments.

*E. coli* isolates were grown at 37˚C in Luria-Bertani (LB) broth, and BAA1117 was grown at 30˚C in marine broth (BD, 2216). In addition, the bacterial growth was determined using LB broth containing 1/4 MIC of CAZ or CFP with rapid shaking at 37˚C. For the growth curve experiments, 50 µl of the culture sample was collected every 4 h for 24 h to measure the optical density at 600 nm with a Thermo Multiskan Spectrum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (8).

**Determination of MICs for *E. coli* strains.** The MICs of the four cephalosporins against the *E. coli* strains were determined in accordance with the Clinical and Laboratory Standards Institute guidelines (9). Cultures were adjusted to a turbidity equivalent to 0.5 MacFarlane standard suspension prior to being inoculated on Mueller Hinton agar (Oxoid, Basingstoke, UK) in the presence of CAZ, ceftriaxone, cefotaxime or CFP at concentrations ranging from 256 to 0.0625 µg/ml (12 doubling-dilution drug concentrations). Cultures were incubated for 24 h at 37˚C under aerobic conditions. The lowest drug concentration that could prevent growth was recorded as the MIC.

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Table I. Oligonucleotides used in reverse transcription-polymerase chain reaction.

| Gene   | Accession no. | Primer       | Sequence (5'-3')                     | Product size (bp) |
|--------|---------------|--------------|--------------------------------------|------------------|
| 16s-rRNA | NC_000913     | 16s-rRNA-F   | ggaggaagttggttggtagcg                | 638              |
|        |               | 16s-rRNA-R   | atggtgtgacggeggtggt                 |                  |
| pfs    | NC_000913     | pfs-F        | cctggcacaacagtgaag                  | 347              |
|        |               | pfs-R        | tggcgegtacacaaacac                  |                  |
| luxS   | NC_000913     | luxS-F       | tgcgaacaaggaagttgac                 | 348              |
|        |               | luxS-R       | ctctttgtgctgtgatgtgacg              |                  |
| ariR   | NC_000913     | ariR-F       | tcaagctgatgagccgacg                 | 156              |
|        |               | ariR-R       | tgcgaacagattcgcg                    |                  |
| hha    | NC_000913     | hha-F        | aatctgttcacgttctggtg                | 135              |
|        |               | hha-R        | ttcattgtgacagtggggtggtg             |                  |
| tnaA   | NC_000913     | tnaA-F       | aactcctgccacatatcgc                 | 248              |
|        |               | tnaA-R       | atcgegcgcttctctcctca                |                  |
| tomB   | NC_000913     | tomB-F       | caacatctggctgtaaaga                 | 265              |
|        |               | tomB-R       | caacagttcttcttttcgtg                |                  |
| mcbR   | NC_000913     | mcbR-F       | egtttctttgcacgctgca                 | 191              |
|        |               | mcbR-R       | gctctgttgcacgctg                     |                  |
| mqsR   | NC_000913     | mqsR-F       | caaacgcggagaagggctg                 | 186              |
|        |               | mqsR-R       | tgcctgtgaacacgtgg                    |                  |
| QseB   | NC_000913     | qseB-F       | cgctgggaaagagggcgtg                 | 258              |
|        |               | qseB-R       | gtgcggcgcctcacacggtt                |                  |
| QseC   | NC_000913     | qseC-F       | actcgcgcttgaacacac                  | 273              |
|        |               | qseC-R       | aagctgttttccgcgcgtg                 |                  |

F, forward; R, reverse; luxS, S-ribosylhomocysteine lyase.
Biofilm formation assay. Biofilm formation was assayed by crystal violet staining of adherent cells as described previously (10), with a few modifications. The bacterial cultures that were adjusted to 1x10^7 cfu/l were inoculated in LB broth on 96-well polystyrene plates in the presence of CAZ or CFP at sub-MICs. Following incubation at 37˚C for 6-24 h, the plates were rinsed twice with phosphate-buffered saline and dried in an inverted position. The adherent cells were stained with 1% crystal violet (Sigma-Aldrich) for 10 min, and the wells were rinsed three times with sterile water. The dye was dissolved in 30% acetic acid, and the absorbance of the solubilized dye at 590 nm was then determined using the Thermo Multiskan Spectrum. Each treatment was assayed in 5-wells per plate and the experiments were repeated three times.

Measurement of mRNA changes of the genes encoding biofilm-modulating proteins. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative (q)PCR were used to investigate the levels of the mRNA products of the biofilm-modulating genes of this isolate. The cultures were inoculated in 20 ml LB broth containing 1/4 MIC of CAZ or CFP and then cultivated at 37˚C with shaking in an environmental chamber (Yuejin, Shanghai, China) for 0.5-10 h. RNA isolation was performed in accordance with the manufacturer's instructions provided for the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RT using a Reverse Transcription system (Promega Corporation, Madison, WI, USA) was conducted as described previously (11). The primers are listed in Table I. RT-PCR and qPCR amplifications were performed. The latter were performed in quadruplet using MyiQ Color Fluorescence Real-Time Quantitative PCR apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The reaction conditions are listed in Table II. For qPCR, 40 cycles were completed and the annealing temperature was 60˚C. The fold changes of each transcript were calculated using the 2^-ΔΔCT method (12) and were expressed as values relative to the control group.

Autoinducer-2 (AI-2) bioassay. The AI-2 bioassay was performed to determine whether AI-2 production in the E42 isolate was affected by CAZ or CFP and to verify whether the gene expression had been inhibited. The bioassays were performed in accordance with the method previously described (13,14). The strains treated with a 1/4 MIC of CAZ or CFP were pelleted by centrifugation at 12,000 x g for 10 min, and then the supernatant was filtered through a
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0.22-µm filter (Millipore, Bedford, MA, USA). *V. harveyi* BB170 was diluted 1:5,000 in fresh Difco Marine Broth 2216 (BD Biosciences, Franklin Lakes, NJ, USA) and then 180 µl of culture was mixed with 20 µl of the supernatant sample. The luminescence values of the mixtures were measured with a luminometer (Turner Biosystems, Sunnyvale, CA, USA) after incubating at 30°C for 3 h. A positive control was obtained from the overnight cultures of BB170 and sterile medium served as a negative control. AI-2 activity was expressed as the difference in relative light units compared with the level of luminescence induced by the negative control. For each experiment, the bioassay was performed in triplicate for each sample.

Transduction of antisense oligonucleotides (AS-ODNs). AS-ODNs targeting S-ribosylhomocysteine lyase (*luxS*) of *E. coli* were designed and used to inhibit gene expression (15,16). Firstly, four AS-ODNs were designed (5'-GGTAATGGTGTAGAGATTATCGATA-3', 5'-CAATGGAAGACGTGCTGAAAGTGCA-3', 5'-GAACGTCTACCAGTGTGGCACTTAC-3' and 5'-CGAAAGAAGAAGTTGCAGGAACTGCA-3'; available at https://rnaidesigner.invitrogen.com/rnaiexpress/) and the one with the highest blocking efficiency was selected for the subsequent experiments. Briefly, 4 µl (20 µg) of the transfection reagent Sofast (5 mg/ml; Sunmabio, Xiamen, China) was diluted in 20 µl LB broth. Following incubation at room temperature for 20 min, 20 µl of AS-ODN was added to the diluted transfection reagent. A total of 40 µl of the mixture was added to 160 µl of the competent bacterial suspension with a final concentration of 10 µM AS-ODN. Following culturing at 37°C for 1 h, the transfected bacteria were adjusted to a turbidity equivalent of a 0.5 MacFarlane standard suspension and cultivated at 37°C with shaking in an environmental chamber for 4 h.

Statistical analysis. The significance of the intergroup differences in terms of biofilm formation, growth or AI-2 production was analyzed using an independent samples t-test. The significance of differences in RT-PCR results was determined using a paired samples t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Biofilm formation by *E. coli* clinical isolate E42 is inhibited by CAZ but induced by CFP. Firstly, the effects of
four third-generation cephalosporins (CAZ, ceftriaxone, cefotaxime and CFP) at 1/4 MICs on the biofilm formation of 52 E. coli isolates were measured. It was identified that CAZ inhibited biofilm formation in seven strains, while CFP enhanced biofilm formation in 18 strains. The results from the E42 isolate were noteworthy as biofilm formation was suppressed by CAZ at 1/4 MIC, whereas it was enhanced by CFP at 1/4 MIC. Therefore, E42 was selected for investigation in subsequent experiments.

CAZ at sub-MICs suppressed biofilm formation by E42 in a dose-dependent manner, and CFP enhanced its biofilm formation in a dose-dependent manner (Fig. 1A). In addition, 1/4 MIC CAZ exerted maximal effects on E42 biofilm formation at 18 h and the effect of CFP at 1/4 MIC reaches a maximum at 24 h (Fig. 1B).

Effects of CAZ and CFP at 1/4 MICs on the mRNA expression of biofilm regulator genes. To determine which regulator gene is involved in the effects of CAZ and CFP on E42 biofilm formation, the changes in the mRNA levels of important regulator genes (pdfs, luxS, arlR, tnaA, hha and tomb) in the bacterial cultures 3 h following inoculation were examined. Among these six genes, the levels of luxS mRNA were associated with CAZ or CFP treatment, whereas mRNA levels of the five other genes were not changed (Fig. 2). The present data suggested that luxS/AI-2 QS may be involved in the inhibitory effects of CAZ and the inductive functions of CFP on E. coli biofilm formation. Therefore, the subsequent experiments focused on luxS/AI-2 QS.

CAZ exerts effects opposite to that of CFP on the mRNA levels of luxS and AI-2 production in E. coli isolates. QS with luxS/AI-2 is known to be important for E. coli biofilm formation (17). Therefore, the mRNA levels of luxS and AI-2 production in the clinical isolate E42 in the presence of 1/4 MICs of CAZ or CFP were further quantified. Considering that AI-2 production is primarily determined by the density of bacterial inoculates, the growth of bacterial cells was examined simultaneously. CAZ and CFP at 1/4 MICs exerted effects on the mRNA levels of luxS and AI-2 production, without any effect on the growth of the isolate (Fig. 3). In E42, the mRNA levels of luxS was significantly reduced following growth in the LB medium with 1/4 MIC CAZ incubated for 30 min or 1 h, compared with the controls in which CAZ was absent. However, these levels were higher following exposure to 1/4 MIC CFP for 0.5-6 h, compared with the controls without CFP (Fig. 3A). Based on the AI-2 bioassay, changes in bioluminescence occurred following treatment for 4 h (Fig. 3B). These results were concurrent with those of the mRNA levels of luxS.

Effects of CAZ and CFP at 1/4 MICs on the mRNA levels of biofilm-modulating factors that are regulated by AI-2. During biofilm formation, AI-2 stimulates biofilm formation and changes its architecture by stimulating flagella motility via MqsR, QseBC and McbR (18). The mRNA levels of key downstream genes that are regulated by AI-2 in the cultures incubated with CAZ or CFP at 1/4 MICs for 12 h. The expression levels of mqsR, qseB, qseC and mcbr in E42 were significantly decreased in the presence of 1/4 MIC of CAZ, whereas CFP exerted the opposite effect on these genes (Fig. 4).

AS-ODNs targeting luxS modulate the effects of CAZ and CFP on E. coli biofilm formation. To confirm the role of luxS in the response to CAZ and CFP, the AS-ODNs targeting luxS were employed. AI-2 production was measured to verify whether luxS expression was blocked. As AS-ODNs do not pass freely through cell membranes due to their negative charge, the cationic polymer reagent Sofast was used to transduce the AS-ODNs, as previously described (15,16). The results demonstrated that the transduction of AS-ODNs targeting luxS decreased AI-2 production significantly, indicating that luxS expression was successfully blocked (Fig. 5A). As AS-ODN-4 (5'-CGAAGAGAGATTGGAGGAACCTGCA-3') was the most efficient at blocking luxS expression, it was selected for the subsequent experiments. The E. coli biofilm formation in the presence of 1/4 MIC of CAZ or CFP was then quantified. Following AS-ODN transduction, the effects of CAZ and CFP on E. coli biofilm formation were diminished (Fig. 5B), suggesting that CAZ and CFP were unable to affect biofilm formation once the luxS gene was blocked.

Discussion

To the best of our knowledge, this is the first study to demonstrate that two antibiotics, CAZ and CFP, sharing the same
The similar antimicrobial activities of CAZ and CFP are due to the similarity of their basic ß-lactam structure (25). It was speculated that their opposite effects on biofilm formation were due to different side chains (26). To determine whether this hypothesis is reasonable, the studies published in the past three years regarding the effects of antibiotics on the biofilm formation of various bacteria were reviewed. However, there was little evidence indicating an association between biofilm-influencing activity and the structures of the antibiotics. Therefore, this hypothesis requires further investigation.

In the present study, the concentrations tested were carefully selected so that the antibiotics did not affect the bacterial growth. Nevertheless, the biofilm formation of the \textit{E. coli} isolate E42 was affected by these low CAZ and CFP doses. Previous studies have demonstrated that the effects of certain antibiotics on the growth and virulence-gene expression of bacteria at higher concentrations than MICs may act via QS (6,17). The results indicate that the response of this isolate to CAZ and CFP at 1/4 MIC may also be due to QS.

In the present study, the biofilm formation of 52 \textit{E. coli} isolates was measured, but only the production of biofilm by E42 responded to CAZ in an opposite manner to that of CFP. This result appears to indicate an individual case. Differences in the reactions among \textit{E. coli} isolates appear to be due to individual differences in isolates. The QS of luxS/AI-2, as an important intermediate, may be involved in the responses to CAZ and CFP, while the underlying mechanisms require further study.

In conclusion, the present study indicates that the effects of antibiotics at sub-MICs may differ from their effects at high doses. The present study does not fully explain the potential effects of these drugs, and therefore extensive and careful investigation is required to determine the clinical application of this data.

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