J Infect Chemother xxx (2018) 1–6

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Sub-minimum inhibitory concentrations of ceftazidime inhibit *Pseudomonas aeruginosa* biofilm formation

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ARTICLE INFO

Article history:
Received 30 November 2017
Received in revised form
27 December 2017
Accepted 13 January 2018
Available online xxx

Keywords:
Sub-minimum inhibitory concentrations
Ceftazidime
Pseudomonas aeruginosa
Biofilm

ABSTRACT

Pseudomonas aeruginosa exhibits the biofilm mode of growth and causes chronic as well as acute infections in humans. Several reports have shown that the treatments with sub-minimum inhibitory concentrations (sub-MICs) of antimicrobial agents influence biofilm formation by *P. aeruginosa*. The antibiotic ceftazidime (CAZ) is used to treat *P. aeruginosa* infections, but few studies have examined the effects of β-lactams on biofilm formation by *P. aeruginosa*. In this study, we investigated the role of sub-MICs of CAZ in the formation of *P. aeruginosa* biofilms. 1/4 × MIC CAZ reduced the biofilm volume of *P. aeruginosa* PAO1, as quantified by crystal violet staining. The formation of *P. aeruginosa* PAO1 biofilms treated with 1/4 × MIC CAZ were observed by confocal laser scanning microscopy. They were more heterogeneous than the PAO1 biofilms without CAZ treatment. Furthermore, sub-MICs of CAZ inhibited the twitching motility, which played an important role in mature biofilm formation. 1/4 × MIC CAZ also reduced the gene expressions of *lecA*, *lecB*, *pel* and *psl*, which mediate the adhesion and polysaccharide matrix synthesis of *P. aeruginosa*. These effects suggest that sub-MICs of CAZ may affect a number of stages of biofilm formation. Investigating the effects of sub-MIC antibiotics on targeted bacterial biofilm may lead to the development of future antibiotic treatment modalities.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium found in common environments and a pathogen responsible for various opportunistic infections, including chronic airway infections. During the course of chronic infection, *P. aeruginosa* forms biofilms, which are matrix-encased, surface-associated microbial communities. Biofilm formation plays important roles in the pathogenesis and contributes to the establishment of chronic pulmonary infections by evasion of the host's immune response [1]. In addition, biofilms are up to a thousand times more tolerant of all antimicrobial agents than their planktonic counterparts [2].

Microscopic analyses have shown that biofilm formation occurs in sequential stages of initial adhesion, formation of microcolonies and biofilm maturation [3]. Polar-localized type IV pili of *P. aeruginosa* play an important role in the bacterial attachment to the surface, followed by twitching motility. It was previously reported that type IV pili mutants did not develop microcolonies and therefore lacked a mature biofilm [4]. In addition, the two lectins LecA and LecB produced by *P. aeruginosa* may mediate the adhesion of *P. aeruginosa* to other cells together with pili [5,6]. Several studies have suggested that the downregulation of *lecA* and *lecB* genes likely contributes to a reduction in the formation of *P. aeruginosa* biofilms [6,7].

Extracellular polysaccharides also carry out important functions for building and maintaining the mature biofilm structure. *P. aeruginosa* produces at least three extracellular polysaccharides: Pel, Psl and alginate. Non-mucoid *P. aeruginosa* strains utilize the Pel and Psl polysaccharides to enhance mature biofilm formation [8], while alginate is overproduced in mucoid strains and also plays an important role in mature biofilm formation. The *pel* locus contains seven genes (*pelABCDEFG*) encoding a range of functions involved in the synthesis of polysaccharide [9] and is necessary for the maintenance of the biofilm structure in *P. aeruginosa* [10]. The *polysaccharide synthesis locus* (*psl*) is an operon required for the

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https://doi.org/10.1016/j.jiac.2018.01.007
1341-321X/

biosynthesis and export of the Psl polysaccharide [8,11]. Psl, which consists of D-glucose, D-mannose and L-rhamnose monosaccharides, is needed for cell-surface and cell-cell interactions in addition to the maintenance of the biofilm structure after attachment.

Several studies have investigated the inhibitory effects of antibiotics against biofilms. The macrolide antibiotic azithromycin retarded *P. aeruginosa* biofilm formation while mature biofilms were not affected [12]. High concentrations of tobramycin reduced the amount of *P. aeruginosa* biofilm formed. Furthermore, synergistic effects between tobramycin and clarithromycin were observed against *P. aeruginosa* biofilm *in vitro* [13]. However, few studies have examined the effects of β-lactams on biofilm formation by *P. aeruginosa*.

In this study, we investigated the antimicrobial effects of sub-minimum inhibitory concentrations (sub-MICs) of ceftazidime (CAZ) on the biofilm formation of *P. aeruginosa* strains by microscopic analysis and quantifying the expression of biofilm-related genes.

2. Materials and methods

2.1. Agents and bacterial strains

Ceftazidime hydrate (Sigma-Aldrich, Tokyo, Japan) was used in this study. The bacterial strains used in this experiments were *P. aeruginosa* wild-type strain PAO1 and *P. aeruginosa* Δ*pilHIJK*, which lacks the ability to produce intact type IV pili and is unable to translocate across surfaces by twitching motility [14]. For the flow chamber experiment, PAO1 and Δ*pilHIJK* mutants genetically tagged with green fluorescent protein (GFP) were used [14]. We confirmed that the GFP-tagged strains showed no phenotypic differences from the parental strains in liquid medium.

The MIC of CAZ was determined for PAO1 wild-type, PAO1-GFP, the Δ*pilHIJK* mutant and the Δ*pilHIJK* mutant-GFP. For all tested strains, the MIC of CAZ was 1.56 μg/ml and we confirmed that 0.39 μg/ml CAZ (1/4 × MIC) had no effect on the growth phase in the medium (data not shown). All strains were stored at −80 °C in Luria-Bertani (LB) broth (Sigma-Aldrich, St. Louis, MO, USA) with 50% glycerol and cultured on LB agar plate (1.5%) (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for overnight before use.

2.2. Quantification of biofilm formation with the Crystal Violet staining method

A single colony of *P. aeruginosa* PAO1 and the Δ*pilHIJK* mutant were placed into 10 ml of LB broth and incubated under shaking for 3 h at 37 °C to yield a density of 10^7 CFU/ml. We used the bacterial suspension as the initial bacterial suspension (IBS). The wells of a 96-well polyvinyl chloride microplates (Nunc, Roskilde, Denmark) were inoculated with 50 μl of IBS in 50 μl of 10% trypticase soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with or without various concentrations of CAZ (0.1, 0.2, 0.39, 0.78 and 1.56 μg/ml) and incubated for 48 h at 37 °C under static conditions. A control well was incubated without CAZ or bacteria.

Crystal Violet staining was performed to quantify the biofilm formation of *P. aeruginosa* as described previously [15]. In brief, the incubated medium was removed from the wells followed by washing with sterile distilled water. The adherent bacterial cells were stained with a 1% Crystal Violet solution for 45 min. The excess Crystal Violet was discarded, and the remaining dye was dissolved with ethanol solution after the wells were washed three times with sterile distilled water. The absorbance of each well was determined at 595 nm in a microplate reader (SPECTRAFluor Plus®; TECAN, Grödig, Austria). Each experiment was evaluated in triplicate, and the results were expressed as the difference in the OD_{595} (OD_{595} sample − OD_{595} control).

2.3. The observation of biofilm formation with microscopy

Biofilms were formed in flow chambers using GFP-tagged PAO1 and Δ*pilHIJK* mutants as described previously [16]. A single colony of each strain was placed into LB broth and shaken for 6 h at 37 °C. Flow chambers with dimensions of 0.4 × 3.8 × 17 mm (μ-slides®, ibidi, Martinsried, Germany) were inoculated by injecting 200 μl of the culture diluted with 10% TSB with or without various concentrations of CAZ (0.2, 0.39 and 1.56 μg/ml) to 10^8 CFU/ml into each channel. After 1 h of inoculation, the flow of medium with or without the CAZ was started at 3 ml/h at room temperature. The biofilm formation was observed by confocal laser scanning microscopy (CLSM) using the LSM5 PASCAL® (Carl Zeiss, Jena, Germany) after flow for 48 h. The experiment was repeated at least three times for each strain and concentration.

2.4. An mRNA assay by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed as previously described [17,18], using PAO1 wild-type grown under static conditions in 10% TBS with 1/4, 1/8, 1/16 × MIC or without CAZ. Total RNA was purified from a 3 ml sample of culture grown for 10 h at 37 °C. In brief, as a first step, 2 vol (6 ml) of RNA protect bacteria reagent (Qiagen, Hilden, Germany) was added to 1 × 10^9 bacterial cells. RNA was extracted with the RNeasy Midi Kit (Qiagen), and residual DNA was removed by incubation with RNase-Free DNase (Qiagen) for 15 min at room temperature. RNA was converted to single-stranded cDNAs using the High Capacity cDNA Archive Kit (Applied Biosystems, Courtaboeuf, France). Real-time PCR was carried out in 96-well plates. The 25-μl reaction mixtures contained 12.5 μl of SYBR Green PCR master mix (Applied Biosystems), 900 nM of each primer and cDNA. The conditions were as follows: 95 °C for 10 min, and 40 cycles at 95 and 60 °C for 60 and 30 s, respectively. The relative quantification of the mRNA was performed by the comparative C_T ($2^{-\Delta\Delta CT}$) method. We used 16S rRNA as an endogenous control [17], and PCR experiment was repeated three times. The PCR primers used for 16S rRNA measurements were 16SF (5′-3′ CAGGATTAGATACCCTGGTA GTCCAC) and 16SR (GACTTAACCCAACATCTCACGACAC), based on the sequence of *P. aeruginosa* 16S rRNA [17]. Transcripts of *pelA*, *pslA*, *lecA* and *lecB* were quantified with the following primer pairs, respectively [19]: pelAF(TGTCGGGTATCTGAAAGAGCAG), pelAR(-GACCGACAGATAGGCGAAGG), pslAF(GTTCTGCCTGCTGTTGTTCATG), pslAR(AGGTAGGGAAACAGGCCCAG), lecAF(TGGAAAGGTGAGGTTCT GGC), lecAR(AATCGACGTTACCTGCCCTG), lecBF(ACCAATAACGCCG TCATCG), lecBR(CTGTACCTTGCCACTGCTGC).

2.5. Twitching motility assay

Twitching motility assays were performed by the inoculation of wild-type or Δ*pilHIJK* mutants *P. aeruginosa*, as previously described [14]. Thin (2-mm-thick) LB agar plates (1.5%) with various concentrations of CAZ were stab inoculated with a toothpick to the bottom of a petri dish. After 48 h of incubation at 37 °C, the light haze zone of growth at the agar-plate interface surrounding the colony was determined to be the twitching motility. Each assay was performed in triplicate.

2.6. Statistical analyses

The data were analyzed using the Prism 5.0 software program (GraphPad Software, La Jolla, CA, USA) and expressed as the

Please cite this article in press as: Otani S, et al., Sub-minimum inhibitory concentrations of ceftazidime inhibit *Pseudomonas aeruginosa* biofilm formation, J Infect Chemother (2018), https://doi.org/10.1016/j.jiac.2018.01.007