Microbial Pathogenesis 114 (2018) 57–62

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Sub-inhibitory concentrations of fluoroquinolones increase conjugation frequency

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ARTICLE INFO

Keywords:
Sub-MIC
Transcriptome sequencing
Ciprofloxacin
Levofloxacin
Conjugation

ABSTRACT

Bacteria are subjected to sub-minimal inhibitory concentrations (sub-MIC) of antibiotics in various niches where the low-dosage treatment plays a key role in antibiotic resistance selection. However, the mechanism of sub-MIC of antibiotics on the resistant gene transfer is largely unknown. Here, we used Escher*ichia coli* SM10λpir in which the RP4 plasmid was chromosomally-integrated as the donor strain, to investigate the effects of sub-MIC of Ciprofloxacin(Cip) or Levofloxacin(Lev) on conjugational transfer of mobilisable plasmid-pUCP24T from SM10λpir to *Pseudomonas aeruginosa*. The results showed that the transfer frequency was significantly increased by treating *E. coli* with sub-MIC of Cip or Lev. To investigate the molecular mechanisms, complete transcriptome sequencing was performed. We found that the sub-MIC of Cip or Lev enhanced the expression of several genes on the RP4 plasmid, which was consistent with the conjugation efficiency. Moreover, the expression of genes associated with SOS response in donor SM10λpir was increased, but had no correlation with conjugation efficiency. These findings suggested that sub-MIC of Cip or Lev may promote conjugational transfer by up-regulating the expression of conjugation associated genes via an SOS-independent mechanism.

1. Introduction

The increasing resistance of microbial pathogens to standard antibiotics is a serious threat to public health. Horizontal Gene Transfer (HGT) plays an important role in the spread of antibiotic resistance [1–4]. Conjugation is one of the most important ways for HGT in prokaryotes, leading to genetic variation within a species and the acquisition of new traits [5–9]. The most important role of conjugation is its ability of mediating the conjugational transfer of resistance plasmids, which leads to the spread of antibiotic-resistant genes among a wide range of bacterial species.

Antibiotics, besides antibacterial function, also result in undesirable effects in microbial populations, including the stimulation of HGT [10–13]. The inhibitory effect of antibiotics to microorganisms was dose- and time-dependent. In order to inhibit or kill bacteria, the medication dosage of antibiotics is calculated according to pharmacokinetic parameters to remain above the minimal inhibitory concentration (MIC). However, it has been reported that during the treatment of microbial infections in practice, the concentration of antibiotics is often lower than the MIC, which is the sub-minimal inhibitory concentrations (sub-MIC). The sub-MIC of antibiotics cannot inhibit the proliferation, in contrast, they change physicochemical properties of microorganisms [14–16], particularly induce unintended effect on the conjugational transfer in various bacteria. Low concentrations of antibiotics (Cip, Erythromycin or beta-lactamagents) increase transfer frequencies of resistance plasmids in *E. coli*, or between different derivatives of *Staphylococcus aureus*, or from *E. coli* to *S. aureus* [17–20], whereas other antibiotics, including vancomycin and teicoplanin, have no such effect. So far, only one study showed the role of quinolones in promoting the transfer of plasmid, while contradictory results have been reported in other studies [21–23]. Therefore, further research is needed to confirm the role of fluoroquinolones in conjugational transfer of drug resistant plasmids.

On the mechanism, studies from Beaber [24] found that SOS response mediated antibiotics-induced horizontal dissemination of resistance genes. The SOS response to DNA damage involves the upregulation of several genes that are critical for the DNA repair and the regulation of cell division which are suppressed by LexA. The SOS response is induced by a variety of environmental factors and antibiotics, for example fluoroquinolones [25]. In this study, we investigated the effect of sub-MIC of Cip or Lev on conjugation and the underlying mechanisms with *E. coli* SM10λpir which integrated plasmid RP4 as

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https://doi.org/10.1016/j.micpath.2017.11.036
Received 20 June 2017; Received in revised form 21 November 2017; Accepted 22 November 2017
Available online 23 November 2017

donor strain and *Pseudomonas aeruginosa* PAO1 as recipient strain. We observed the global transcription pattern, including the SOS response of donor's genome was altered by the treatment with sub-MIC of Cip or Lev. The results showed elevated expressions of several genes in the RP4 plasmid, resulting in increased *E. coli-P. aeruginosa* conjugation frequency. The findings revealed the potential mechanism of antibiotics to promote conjugational transfer and enhance the spread of antibiotic resistance genes.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli SM10λpir [26] strain which contains a mobilisable plasmid pUCP24T was grown in Luria Broth (LB) medium supplemented with Gentamicin at 35 °C with agitation. *P. aeruginosa* (PAO1) strain was grown in LB medium at 35 °C with agitation.

2.2. MIC determinations

MICs were determined by the broth micro-dilution method according to CLSI protocol M07-A8. The volume of broth in the well was 0.1 ml and the volume of inoculums was 0.01 ml (5×10^6 CFU/ml). MICs were interpreted visually after incubation at 35 ± 2 °C for 16–20 h.

2.3. Growth curve

E. coli SM10λpir strain was grown at 35 °C in LB liquid medium with continuous shaking. The bacterial culture was concurrently replenished with different sub-MIC of Cip or Lev. The bacterial culture was collected after 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, and 13 h of inoculation and measured at OD_{600}.

2.4. In vitro conjugation experiments

Conjugation experiments were carried out as described by Schmidt [27] with some modifications. Donor *E. coli* SM10λpir and recipient PAO1 (gift from Barbara H. Iglewski.) were incubated in LB medium for 8 h with shaking at 35 °C. Each batch of culture was centrifuged at 14,000 × g for 2 min and pellets were resuspended in the same volume of LB liquid medium. Each mating pair suspension was mixed and placed on the surface of a sterile 0.22 μm filter (Millipore). These filters were incubated on LB plates for 2 h. Then, the filters were transferred to a clean container and washed with a 10 ml medium by vortexing the filter. In order to screen transconjugants, 100 μl of the suspensions were then plated on the selective LB plates containing Gentamicin (30 μg/ml) and Ampicillin (100 μg/ml) and incubated for 24 h at 35 °C. The total number of donors was counted by Sysmex UF-1000i (The fully automated urine particle analyzer UF-1000i of Sysmex). Conjugation frequencies were calculated by dividing the number of transconjugants by the number of *E. coli* SM10λpir donors. Conjugation frequencies were determined in three to six independent tests.

2.5. Isolation of total RNA

Total bacterial RNA was isolated from 4 ml of the *E. coli* SM10λpir cultures after 8 h of antibiotic treatment by using SV Total RNA Isolation kits (NO.Z3100 Promega), according to the recommendations of the manufacturer.

2.6. Transcriptome resequencing

Entire sequencing was conducted by BGI Company. The rRNA was removed with kit. Then mRNA was interrupted into short fragments. Taking these small fragments as templates, the first strand and the second strand of cDNA were synthesized respectively. Those short fragments were purified and added with poly (A). After that, the short fragments were connected with the sequencing adapters. The second strand was then degraded by UNG enzyme. The product was purified before PCR amplification. At last, the library could be sequencing using Illumina HiSeq2000. The expression of Unigene was calculated by RPKM method (Reads Per kb per Million reads). The genes between two samples that satisfied the following conditions were identified as differentially expressed genes (≥2-fold differential expression change in treatment conditions versus normal controls, $P < 0.05$, false discovery rate (FDR) ≤ 0.001).

2.7. GO analysis

All Differentially Expressed Genes (DEGs) were mapped to the GO entries in the database (http://www.geneontology.org/). The number of genes in each item was calculated. The hyper geometric test was utilized to identify the significant enriched GO background in the DEGs relative to the genome background. The posthoc adjust *P*-value were calculated by Bonferroni Correction. The GO term with adjusted-*P* value ≤ 0.05 was defined as significantly enriched GO terms in DEGs.

2.8. Quantitative RT-PCR

Total RNA was extracted using the total RNA isolation reagent (Promega, USA). The purified RNA (1 μg) was reverse transcribed to cDNA using the PrimeScript RT reagent kit (TaKaRa, China). Relative real-time PCR was performed on the ABI ViiA™ 7Dx system (Applied Biosystems, USA) using SYBR green I dye chemistry (Termo Sientific) for qPCR detection of the cDNA. The housekeeping gene ribosomal protein rpoD was used as an internal control for normalization. The relative changes in gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Sequences for primers were listed as follows

TraI forward: 5′GACGAGCAAGGCAAGACCG-3′;
TraI reverse: 5′CGGAAGCTAACCAGCAGGTGATA-3′;
TraK forward: 5′GCTGCGGTTGCGTTCCTG-3′;
TraK reverse: 5′ACTTGACCTTCCCCGTTTCC-3′;
RpoD forward: 5′CGTGGTAAGGAGCAAGGCTATC -3′;
RpoD reverse: 5′TCACCTGAATGCCCATGTCG-3′

2.9. Statistical analysis

Data were represented as mean ± SD from seven samples from three independent experiments. Differences between the mean were analyzed by one-way analysis of variance (ANOVA, STATISTICA 9 PL). Statistical significance was considered when *P* was ≤0.05.

3. Results

3.1. Conjugation frequency was enhanced by sub-MIC of Cip or Lev

In order to study whether the conjugation between *E. coli* and *P. aeruginosa* is affected by sub-MIC of Cip or Lev, the MICs on the donor treated with these two drugs were firstly determined as 0.125 μg/ml (Cip) and 0.25 μg/ml (Lev) by the broth micro-dilution method. We next confirmed the MICs through growth curve analysis. It could be observed that the growth curve was disturbed by high concentrations of antibiotics (1/2 MIC and 1/4 MIC), whereas was only slightly affected by lower sub-MIC of Cip or Lev (1/8 MIC to 1/32 MIC) (Fig. 1). Since it has been reported that bacterial conjugation efficiency is closely related to the growth state of bacteria [28,29], we chose 1/8 MIC to 1/32 MIC which has no effect on bacteria growth to study the effect of sub-MIC of Cip or Lev on conjugation.

The donors were pre-grown separately under the indicated sub-MIC of antibiotics for 8 h and then subjected to conjugation experiment. For