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ORIGINAL ARTICLE

Restoration of antibiotic susceptibility in fluoroquinolone-resistant *Escherichia coli* by targeting *acrB* with antisense phosphorothioate oligonucleotide encapsulated in novel anion liposome

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Fluoroquinolone-resistant *Escherichia coli* (FREC) is one of the leading causes of Gram-negative bacterial infections short of effective antibiotics, thus necessitating development of novel antibacterial agents such as antisense resistance inhibitors. Aiming to restore susceptibility of FREC to fluoroquinolones by antisense inhibition of essential resistance mechanism, we designed and synthesized anion liposome encapsulated phosphorothioate oligodeoxynucleotide 831 (PS-ODN831) targeting gene *acrB*, which encodes the AcrAB–TolC efflux pump responsible for decreasing intercellular antibiotic concentrations. In all encapsulated PS-ODN831-treated groups, the MICs of ciprofloxacin and levofloxacin to FREC were reduced at different degrees, therefore inhibiting growth of FREC in a concentration-dependent manner. Reversion of their bactericidal effects was the result of specific and potent inhibition of *acrB* mRNA and the activity of efflux pump of AcrAB–TolC in FREC strains by liposome-encapsulated PS-ODN831. The study indicated that antisense targeting of AcrAB–TolC efflux pump system may be a feasible and potential strategy to treat FREC infections.
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Keywords: *acrB*; anionic liposome; antisense antibacterial strategy; efflux pump; fluoroquinolone-resistant *Escherichia coli* (FREC); phosphorothioate oligodeoxynucleotide (PS-ODN)

INTRODUCTION

Escherichia coli is the most common etiologic agent of Gram-negative bacilli and often causes extraintestinal infections in its hosts.[1] Infections caused by *E. coli* are routinely treated with quinolones and fluoroquinolones, which belong to the most-frequently prescribed antibiotic classes.[2,3] Quinolones and fluoroquinolones, with their enhanced systemic activity against many Gram-negative bacteria, have been widely used since the first introduction of ciprofloxacin in 1987.[4] Unfortunately, as the use of fluoroquinolones increases, fluoroquinolone therapies are also threatened by an increasing prevalence of fluoroquinolone-resistant *E. coli* (FREC).[5,6] In Beijing, from 1997 to 1999, approximately 60% of *E. coli* strains isolated from hospital-acquired infections and 50% of those isolated from the community were resistant to ciprofloxacin.[7] In Japan, the isolation frequency of levofloxacin-resistant clinical isolates of *E. coli* increased from 14.6 to 20.8% from 2003 to 2008.[8] According to the European resistance surveillance network, a significant increase of fluoroquinolone resistance was observed for *E. coli* between 2002 and 2009, and the majority of countries reported resistant proportions to be >20%, reaching up to 43.4%. Although the number of FREC strains is increasing all over the world, no new antibiotics are under development to provide plausible therapeutic choices.[9] Thus, it is urgently needed that novel alternative strategies with mechanisms radically different from the existing ones can be used to combat FREC.

The concept of using the antisense approach to combat antibiotic-resistant bacteria is revolutionary. Antisense oligomers target specific genes and inhibit transcription and expression of the targeted sequence. Sequence data of the entire genome of pathogenic bacteria or of multiple isolates of a single pathogen have provided new insights into the microevolution of a species as well as newly generated targets for antimicrobials.[10] Base sequence-specific antisense antibiotics are now more attractive than ever. They can be rapidly synthesized, targeting any gene with known sequence and being structurally modified to overcome any resistance that may arise.[11] Our previous results have demonstrated that the blockage of resistant gene expression of methicillin-resistant *Staphylococcus aureus* and multidrug-resistant *Pseudomonas aeruginosa* by phosphorothioate oligodeoxynucleotide (PS-ODN) or deoxyribozyme leads to significant reduction of resistant gene mRNAs. As a result, the susceptibility of methicillin-resistant *S. aureus* and multidrug-resistant *P. aeruginosa*

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to some antibiotics has been restored to a significant level.[12–16] Fluoroquinolone resistant clinical strains of *E. coli* frequently show an upregulated AcrAB–TolC system in addition to alterations in targets DNA gyrase and Topoisomerase IV, which suggests that the pump may provide some advantages in the clinical setting.

In the current study, we used the specific anti-*acrB* antisense PS-ODN for inhibiting the expression of *acrB* mRNA, thereby restoring the susceptibility of *E. coli* to fluoroquinolones.

RESULTS

Reversal of antibiotic resistance of FREC070701 by encapsulated anti-*acrB* PS-ODN831

We found that the number of FREC070701 colonies on the Mueller–Hinton agar containing ciprofloxacin ($6\,\mu g\,ml^{-1}$) or levofloxacin ($12\,\mu g\,ml^{-1}$) decreased significantly in all envelopment anti-*acrB* PS-ODN831 liposome-treated groups in a concentration-dependent manner. When FREC070701 strain was treated with liposome-encapsulated $100\,\mu g\,ml^{-1}$ anti-*acrB* PS-ODN831, the number of colonies in Mueller–Hinton agar containing $6\,\mu g\,ml^{-1}$ of ciprofloxacin decreased from 5.15×10^9 to 1.2×10^8 cells ml^{-1} and the number of colonies in Mueller–Hinton agar containing $12\,\mu g\,ml^{-1}$ of levofloxacin decreased from 3.88×10^9 to 1.42×10^8 cells ml^{-1}. The number of FREC070701 colonies on the Mueller–Hinton agar containing ciprofloxacin ($6\,\mu g\,ml^{-1}$) decreased to 33.4, 9.9, 6.6 and 2.3% of the control value in all encapsulated PS-ODN831-treated groups at 3, 10, 30 and $100\,\mu g\,ml^{-1}$, respectively (Figure 1a). Similarly, we also observed that compared with the control group, the number of FREC070701 colonies from all anti-*acrB* liposome-encapsulated PS-ODN831 at 3, 10, 30 and $100\,\mu g\,ml^{-1}$ on the Mueller–Hinton agar containing levofloxacin ($12\,\mu g\,ml^{-1}$) significantly decreased to 34.8, 21.2, 7.0 and 3.7%, respectively, of the control value (Figure 1b). However, the growth of FREC070701 was not influenced in the liposome-encapsulated PBS, liposome-encapsulated mismatched PS-ODN0701 ($100\,\mu g\,ml^{-1}$), free PEI-treated group ($5.5\,\mu g\,ml^{-1}$), but was only slightly inhibited in the free PS-ODN831-treated group ($100\,\mu g\,ml^{-1}$), which indicated that a small amount of free PS-ODN831 could enter into the bacteria through the cell wall of bacteria (Figures 1a and b).

Additional experiments were performed to determine growth rate of FREC070701 in ciprofloxacin ($6\,\mu g\,ml^{-1}$) or levofloxacin ($12\,\mu g\,ml^{-1}$) containing liquid medium (Figure 2). The results showed that the growth of FREC070701 was appreciably inhibited when it was treated with different concentrations of encapsulated anti-*acrB* PS-ODN831 liposome concentration-dependently as compared with cells grown in control group (Figures 2b and d). However, the growth of FREC070701 in ciprofloxacin ($6\,\mu g\,ml^{-1}$) or levofloxacin ($12\,\mu g\,ml^{-1}$) containing liquid medium was not influenced by the treatment with liposome-encapsulated PBS, liposome-encapsulated mismatched PS-ODN0701 ($100\,\mu g\,ml^{-1}$), free PEI ($5.5\,\mu g\,ml^{-1}$) and free PS-ODN831 ($100\,\mu g\,ml^{-1}$) (Figures 2a and c).

Restoration of antibiotic susceptibility of FREC070701 to clinical commonly used antibiotics ciprofloxacin and levofloxacin

Furthermore, we observed the change of MIC of ciprofloxacin and levofloxacin against FREC070701 in the presence or absence of encapsulated anti-*acrB* PS-ODN831 liposome. FREC070701 showed the strong resistance to ciprofloxacin and levofloxacin. The MICs of ciprofloxacin and levofloxacin were 64 and $32\,\mu g\,ml^{-1}$, respectively. The MICs of ciprofloxacin to FREC070701 were reduced at different degrees in the presence of liposome-encapsulated PS-ODN831 at 3, 10, 30 and $100\,\mu g\,ml^{-1}$. After treatment with $100\,\mu g\,ml^{-1}$ liposome-encapsulated PS-ODN831, the MIC of ciprofloxacin to FREC070701 was decreased from 64 to $1\,\mu g\,ml^{-1}$, which reached sensitive margin values of ciprofloxacin to *E. coli* on the basis of the interpretive criteria recommended by the Clinical and Laboratory Standards Institute. At the same time, the MICs of levofloxacin to FREC070701 were also reduced at different degrees when treated with 3, 10, 30 and $100\,\mu g\,ml^{-1}$ encapsulated PS-ODN831 liposome. After treatment with $100\,\mu g\,ml^{-1}$ liposome-encapsulated PS-ODN831, the MIC of levofloxacin to FREC070701 was decreased from 32 to $1\,\mu g\,ml^{-1}$. The MIC values of ciprofloxacin and levofloxacin to FREC070701 in the presence of free anti-*acrB* PS-ODN831 were only decreased a little. However, the MIC values of ciprofloxacin and levofloxacin for liposome-encapsulated mismatched PS-ODN0701 ($100\,\mu g\,ml^{-1}$), free PEI ($5.5\,\mu g\,ml^{-1}$) and liposome-encapsulated PBS group remained unchanged (Table 1).

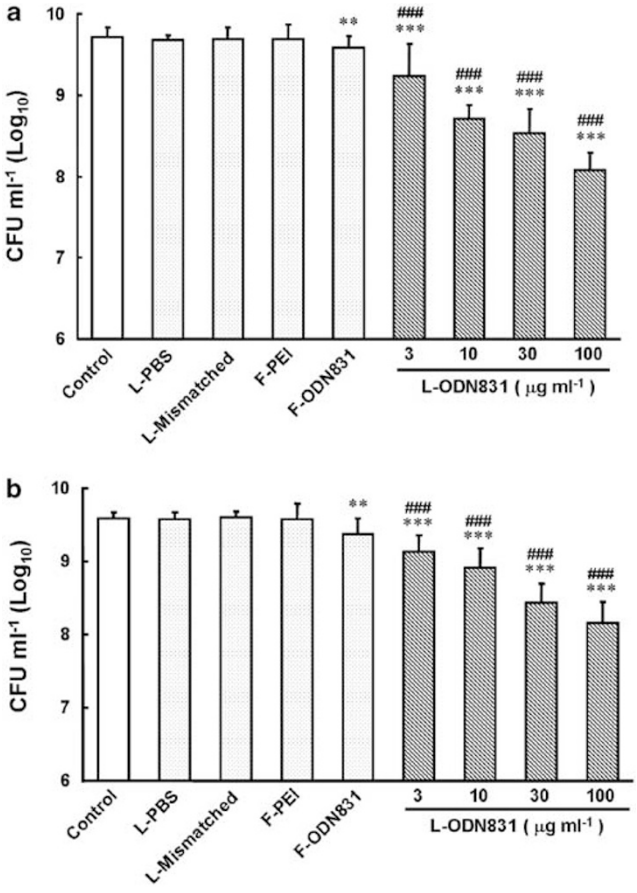

Figure 1 Effects of anti-*acrB* encapsulated PS-ODN831 on the growth of FREC070701 colonies. (**a**) Colony forming unit (CFU) of FREC070701 on Mueller–Hinton agar containing $6\,\mu g\,ml^{-1}$ of ciprofloxacin. (**b**) CFU of FREC070701 on Mueller–Hinton agar containing $12\,\mu g\,ml^{-1}$ of levofloxacin. The data were shown as mean ± s.d. of 10 samples, $^{**}P<0.05$ vs control, $^{***}P<0.01$ vs control, $^{\#\#\#}P<0.01$ vs free PS-ODN831.

Real-time quantified assays for *acrB* expression

We next, determined whether the restoration of antibiotic susceptibility in FREC070701 was correlated with the downregulation of *acrB* by the addition of anti-*acrB* PS-ODN831. To ascertain whether anti-*acrB* PS-ODN831 inhibits expression of target gene, the encapsulated anti-*acrB* PS-ODN831 liposomes were added to FREC070701 and the expression of *acrB* mRNA of FREC070701 was detected using real-time PCR.

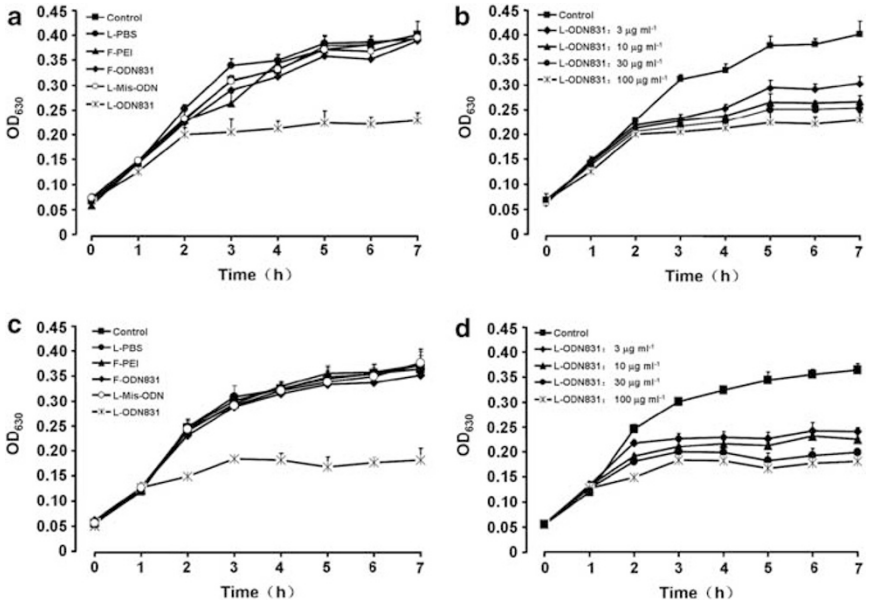

Figure 2 Effect of anti-*acrB* encapsulated PS-ODN831 on growth of the FREC070701 in liquid culture medium. The cells were cultured in liquid medium containing 6 μg ml^{-1} of ciprofloxacin (**a** and **b**) or 12 μg ml^{-1} of levofloxacin (**c** and **d**). The data were shown as mean ± s.d. of 10 samples.

Table 1 MICs of ciprofloxacin and levofloxacin for clinically isolated strain FREC070701 in the presence or absence of anti-*acrB* PS-ODN831

Groups	Concentrations of PS-ODN831(μg ml^{-1})	MICs (μg ml^{-1})	
		Ciprofloxacin	Levofloxacin
Control	0	64	32
L-PBS	0	64	32
L-Mis-ODN	100	64	32
F-PEI	5.5	64	32
F-ODN831	100	32	16
L-ODN831	3	32	8
L-ODN831	10	16	4
L-ODN831	30	4	2
L-ODN831	100	1	1

The MIC of ciprofloxacin to *Escherichia coli*: sensitive: MIC ⩽ 1 μg ml^{-1}; resistant: MIC ⩾ 4 μg ml^{-1}. The MIC of levofloxacin to *E. coli*: sensitive: MIC ⩽ 2 μg ml^{-1}; resistant: MIC ⩾ 8 μg ml^{-1}.

Table 2 Oligonucleotide primers used for real-time PCR

Gene	Primers	Primer sequence(5′-3′)	Location (nucleotide)	Size(bp)
AcrB	Forward	5′-TGGCGAGCAAACTGCCTAC-3′	2540–2560	126
	Reverse	5′-CCAGACACAGGAACACGACAA-3′	2665–2685	
16SrRNA	Forward	5′-AGCGCAACCCTTATCCTTTGT-3′	1095–1115	85
	Reverse	5′-ATCCCCACCTTCCTCCAGTT-3′	1179–1199	

Melting curve analysis is a simple, straightforward method to check qPCR reactions for primer–dimer artifacts and contamination, and to ensure reaction specificity and accurate quantification. Melting curve analysis demonstrated that each of the primer pairs, as described in Table 2, amplified a single predominant product with a distinct melting temperature. The melting temperature of *acrB* was 84 °C and the melting temperature of *16SrRNA* was 83 °C. This was confirmed by running 10 μl of each product on an ethidium bromide-stained 1% agarose gel. The negative controls did not show any amplification product (data not shown).

To demonstrate that the PCR efficiencies for the target and the control gene were approximately equal, the values of the two standard curves were used to determine the absolute value of the slope of the log5 cDNA versus ΔCt (difference in the cycle threshold obtained for the two PCR systems with the same cDNA dilution) for the respective dilution. This validation experiment involved pairwise comparisons between *acrB* and *16SrRNA* and the slope was 0.0023 (Figures 3a and b), demonstrating approximately equal PCR amplification efficiencies between *acrB* and *16SrRNA*.

Using the $2^{-\Delta\Delta Ct}$ method, the change in gene expression of *acrB* mRNA in FREC070701 was normalized to *16SrRNA* mRNA. Compared with the control group, the relative expression of *acrB* in anti-*acrB* PS-ODN831-treated groups (3, 10, 30 and 100 μg ml^{-1}) was decreased in a concentration-dependent manner to 67, 36, 17 and 13% of control values, respectively. The relative expression of *acrB* in free PS-ODN831 group was 85% compared with the control group. Moreover, no detectable changes were observed in the liposome-encapsulated PBS, liposome-encapsulated mismatched PS-ODN and free PEI-treated groups (Figure 3c). This result demonstrates that the encapsulated PS-ODN831 not only enters the bacterial cells, but also interacts with the *acrB* mRNA.

DISCUSSION

The fluoroquinolone class of antimicrobial agents has been widely accepted in treating hospitalized and community patients for the treatment of infection caused by *E. coli*. With the increasing use of quinolones, there have been many reports on the increasing incidence of quinolone resistance in *E. coli*.[2,5] The monitoring result of global SMART central indicated that the susceptibility rate of *E. coli* to

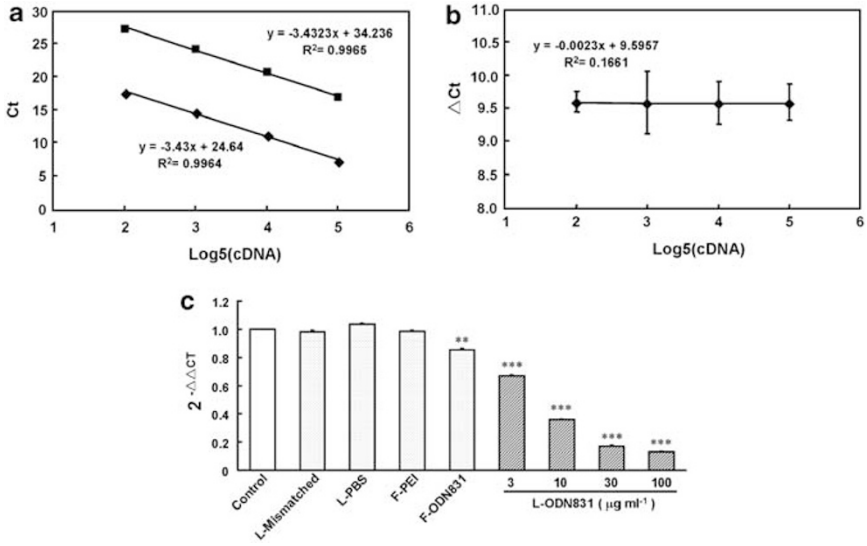

Figure 3 Relative mRNA expression of *acrB* of FREC070701 detected by real-time PCR. (**a**) Standard curves for *acrB* and *16SrRNA* using cDNA from control. (**b**) Relative PCR efficiency of amplification for *acrB* and *16SrRNA* using the respective primers. (**c**) Relative mRNA expression of *acrB* of FREC070701. $\Delta\Delta C_t=(C_{t\cdot acrB}-C_{t\cdot 16S})_{treatment}-(C_{t\cdot acrB}-C_{t\cdot 16S})_{control.}$ (mean ± s.d., n=3). $^{**}P<0.05$ vs control and $^{***}P<0.01$ vs control.

ciprofloxacin and levofloxacin compared with the other agents tested was the lowest in Asia-Pacific region including China in 2005.[17]

Antisense oligonucleotides are in theory designed to specifically modulate the transfer of the genetic information to protein. On the basis of mechanism of action of antisense, oligonucleotide can be discerned two classes: (a) the RNase H-dependent oligonucleotides, which induce the degradation of mRNA; and (b) the steric-blocker oligonucleotides, which physically prevent or inhibit the progression of splicing or the translational machinery. Oligonucleotide-assisted RNase H-dependent reduction of targeted RNA expression can be quite efficient, reaching 80–95% downregulation of protein and mRNA expression. Furthermore, in contrast to the steric-blocker oligonucleotides, RNase H-dependent oligonucleotides, such as phosphorothioate oligonucleotides, can inhibit protein expression when targeted to widely separated areas in the coding region.[18–20]

Mechanisms in fluoroquinolone resistance of *E. coli* fall into two principal categories: alterations in drug targets (for example DNA gyrase or topoisomerase IV)[21–26] and decreased cellular accumulation of quinolones. The latter involves the major and constitutively expressed multi-drug efflux pump, AcrAB-TolC.[27,28] The drug proton antiporter AcrB, which is located in the inner membrane, captures its substrates within the phospholipid bilayer[29] and transports them into the external medium via the OM channel TolC.[30] The cooperation of AcrB and TolC is mediated by the periplasmic protein AcrA, which has a large periplasmic domain.[31,32] As the three components are required for efficient transport of drugs, disruption of one of the three proteins by chemical compounds results in hypersusceptibility of *E. coli* to various antibiotics.[33] Similarly, inhibition of AcrAB-TolC at RNA level is likely to be an effective approach to enhance the susceptibility of FREC.

Traditional antibiotics are increasingly suffering from the emergence of multidrug resistance among pathogenic bacteria. A range of novel approaches to control microbial infections is being investigated as potential alternative treatments. An antimicrobial alternative could be the combination of conventional antimicrobial antibiotics with efflux pump inhibitors, which block multidrug efflux systems. Efflux pump inhibitors could increase intracellular concentration of an antibiotic and restore its antibacterial activity of the antibiotic.[34,35] A new potential approach to inhibit efflux pump is the use of synthetic antisense oligonucleotides, which are complementary and antisense relative to mRNA, leading to inhibition of gene product synthesis. The development of antisense oligonucleotides has shown some promise to reverse antibiotics resistance.[36–38] Interrupting expression of resistance genes in this manner could restore susceptibility to key antibiotics, which would be co-administered with the antisense compound. This would extend the lifespan of existing antibiotics, which offer clinically proven therapies, and are often cheaper, more effective or less toxic than the alternatives.[11]

In our study, liposome-encapsulated anti-*acrB* PS-ODN831 reduced the expression of *acrB* gene of FREC070701 strain in a concentration-dependent manner. And when FREC070701 strain was treated with PS-ODN831, ciprofloxacin and levofloxacin could remarkably inhibit growth of FREC070701 strain. So we concluded that through inhibiting *acrB* mRNA expression, PS-ODN831 could decrease the expression of the AcrB protein and inhibit the drug efflux activity of *E. coli* strains. This method might provide a feasible strategy to recover antibiotic susceptibility in fluoroquinolone–resistant *E. coli,* and *acrB* mRNA may prove to be a promising drug target to combat FREC infections in the near future.

METHODS

Chemicals

Levofloxacin and ciprofloxacin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Polyethylenimine (PEI, 25 kDa) was purchased from Aldrich-Sigma (St Louis, MO, USA). Egg yolk phosphatidylcholine was obtained from Xi'an Libang Pharmaceutical Co., Ltd (Xi'an, China). *N*-(carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PEG_{2000}-DSPE) was purchased from HeliCal Technology Ltd (Vancouver, BC, Canada). Dimyristoyl phosphatidylglycerol (DMPG) was purchased from Lipoid (Ludwigshafen, Germany). All culture media were purchased from Beijing Land Bridge Technology Co., Ltd (Beijing, China). All other chemicals and solvents were of analytical grade quality.

Organisms

The *E. coli* clinical strain FREC070701, which was resistant to ciprofloxacin and levofloxacin used in this study, was obtained from patients in Xijing Hospital (Xi'an, China). The strain expressed *acrB*, which is confirmed by PCR detection, and the mutation of DNA gyrase or topoisomerase IV was not detected (data not shown). We also used the laboratory strain *E. coli* ATCC25922 as the test organism and reference strain according to the quality control measures.

Antisense oligonucleotides

We designed a series of PS-ODNs targeting gene *acrB* of *E. coli* strain with RNAstructure 4.6 software (Mathews Lab, Rochester, NY, USA). The identification of the sensitive region of gene *acrB* and optimization of anti-*acrB* PS-ODNs for efficacious gene-specific antisense inhibition were verified by modified MIC assay of PS-ODNs (Supplementary Table S1). The sequence of the most active PS-ODN831 among these oligonucleotides in this study was: 5′-GATGTCGTAGTTCTCACC-3′ (PS-ODN831), which is complementary in sequence to nucleotide 814–831 in the coding region *acrB* mRNA in strain FREC070701. The control-mismatched sequence of this antisense PS-ODN0701, which was randomly aligned with the same number of bases, is 5′-CGAGTCCCTTTTTACCAA-3′. The PS-ODNs were synthesized by Aoke Biotechnology Limited-liability Company (Beijing, China) and were fully phosphorothioated.

Preparation of anionic liposome encapsulated with nano-sized PS-ODN831/PEI complexes

In order to increase encapsulation efficiencies of anionic liposome, PS-ODN831 and PEI were condensed into PS-ODN/PEI nanometer particle, and then this nanometer complexes were enveloped into liposome. Nanometer PS-ODN/PEI complexes and anionic liposome were prepared as described previously.[39] The encapsulation efficiency was found to be up to 80%.

Bacterial growth assay

The bacterial strain FREC070701 was cultured for 12 h (OD_{630}=0.5∼0.6) and then cells were diluted to a concentration of 0.5×10^8 c.f.u ml^{-1}. The diluted bacterial broth was mixed with liposome-encapsulated PBS, liposome-encapsulated mismatched PS-ODN0701 (100 μg ml^{-1}), free PEI (5.5 μg ml^{-1}), free PS-ODN831 (100 μg ml^{-1}) or liposome-encapsulated PS-ODN831 (3, 10, 30 or 100 μg ml^{-1}). The mix cultures were incubated at 37°C with moderate agitation (210 r.p.m.) for 6 h. Then the cultures were diluted in a suitable gradient and 50 μl of diluted cells were spread onto Mueller–Hinton agar which contained 6 μg ml^{-1}of ciprofloxacin or 12 μg ml^{-1} of levofloxacin. Plates were incubated for 48 h at 37 °C. The number of colonies was counted for plates with >10 and <500 colonies. The total colony-forming unit (CFU) per sample was determined by correcting the colony count from the dilution factor.

To determine the FREC070701 growth in the broth medium, 0.5×10^8 c.f.u ml^{-1} cell dilution was mixed with liposome-encapsulated PBS, liposome-encapsulated mismatched PS-ODN0701 (100 μg ml^{-1}), free PEI (5.5 μg ml^{-1}), free PS-ODN831 (100 μg ml^{-1}) or liposome-encapsulated PS-ODN831 (3, 10, 30 or 100 μg ml^{-1}). In all, 100 μl mixture containing 6 μg ml^{-1} of ciprofloxacin or 12 μg ml^{-1} of levofloxacin was added into a 96-well microtiter plate and the culture was incubated at 37°C with 210 r.p.m. agitation. The OD of each well was measured at different time points with a microplate reader (Bio-Rad laboratories, Tokyo, Japan) at 630 nm.

Bacterial susceptibility testing

The MICs of ciprofloxacin and levofloxacin for FREC070701 were determined by a two-fold dilution tube method according to the guidelines of the Clinical and Laboratory Standards Institute. Quality control was assured by *E. coli* ATCC 25922 in every batch. Briefly, serial dilutions of antibiotics in Mueller–Hinton broth were prepared and the bacterial culture was mixed with liposome-encapsulated PBS, liposome-encapsulated mismatched PS-ODN0701 (100 μg ml^{-1}), free PEI (5.5 μg ml^{-1}), free PS-ODN831 (100 μg ml^{-1}) or liposome-encapsulated PS-ODN831 (3, 10, 30 or 100 μg ml^{-1}). The mixture (50 μl) was then added to each tube to achieve a final inoculum of 5×10^5 cells ml^{-1}. Incubation was made at 37°C for 24 h. After incubation, 100 μl of 1% triphenyl tetrazolium chloride, a colorimetric indicator, was added into each tube and cells were further incubated for 3 h at 37 °C. The lowest concentration of antibiotics that resulted in complete inhibition of bacterial growth was indicated by the tetrazolium chloride-based MIC. Lack of bacterial growth showing no color change from the colorimetric indicator would demonstrate that antibiotics had an inhibitory effect.

RNA isolation and reverse transcription

The total RNA was extracted from the bacterial culture by using an RNAprep Tissue/Bacteria kit (Tiangen biotech Co.Ltd, Beijing, China) according to the manufacturer's instructions. The cDNA of *acrB* was synthesized by reverse transcription from 1 μg of each RNA sample. The cDNA was stored at −20 °C.

Real time PCR reaction

The resulting cDNA was amplified by real-time PCR (BIOER line-gene K, Bioer Technology Co., Ltd, Hangzhou, China) using the gene-specific oligonucleotide primers (Table 2). All primers were synthesized commercially (Takara biotechnology Co., Ltd, Dalian, China). The real time PCR was run using SYBR Green I (Takara biotechnology Co., Ltd). The PCR reagents consisted of 12.5 μl of 2×SYBR Premix Ex Taq (Takara biotechnology Co., Ltd), 1 μl of each primer (5 μM) and 5 μl of sample cDNA, in a final volume of 25 μl. Each plate included its own negative controls, where all the reaction reagents except for cDNA were used. The thermal cycling conditions were an initial denaturation step at 95°C for 2 min, 40 cycles at 94°C for 30 s, 56°C for 20 s and 72°C for 30 s. The melting curves of the PCR products were acquired by stepwise increase of the temperature from 70–94°C.

Comparative calculation and determination of relative expression levels of *acrB* of different treated groups

Relative expression of *acrB* mRNA was calculated using the comparative ΔΔCt method. For the ΔΔCt calculation to be valid, the amplification efficiencies of the target gene and reference gene must be approximately equal. A valid method for assessing if two amplicons have the same efficiency was to look at how ΔCt varies with template dilution. The cDNA of control group was five-fold series diluted and the analysis was performed as follows: for each sample, a difference in the cycle threshold values (ΔCt) was calculated for target gene (*acrB*) by taking the mean Ct of duplicate tubes and subtracting the mean Ct of the duplicate tubes for reference gene (*16SrRNA*) measured on an aliquot from the same RT reaction. ΔCt=Ct(*acrB*)−Ct(*16SrRNA*). A plot of the log5 cDNA dilution versus ΔCt was made. If the absolute value of the slope is close to zero, the efficiencies of the target gene and reference gene are similar, and the ΔΔCt calculation for the relative quantification of target may be used. The ΔΔCt values were calculated using the following equation: ΔΔCt=ΔCt (treatment)−ΔCt (control). The ΔCt for the treated sample was then subtracted from the ΔCt for the untreated, control sample to obtain a ΔΔCt. The mean of these ΔΔCT measurements was then used to calculate expression of *acrB* ($2^{-\Delta\Delta Ct}$) relative to *16SrRNA* and normalized to the untreated control as follows: Relative expression=$2^{-\Delta\Delta Ct}$. Analyses of gene expression data using the $2^{-\Delta\Delta Ct}$ method have been reported in the literatures.[40,41] The evaluation of $2^{-\Delta\Delta Ct}$ indicates the change in gene expression relative to the untreated control.

Statistical analysis

Values are expressed as mean ± s.d. and one-way ANOVA analysis followed by the SNK *t*-test. A value of $P<0.05$ is considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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