Short communication: The role of autoinducer 2 (AI-2) on antibiotic resistance regulation in an *Escherichia coli* strain isolated from a dairy cow with mastitis

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ABSTRACT

Extended spectrum β -lactamase (ESBL)-positive *Escherichia coli* is a major etiological organism responsible for bovine mastitis. The autoinducer 2 (AI-2) quorum sensing system is widely present in many species of gram-negative and gram-positive bacteria and has been proposed to be involved in interspecies communication. In *E. coli* model strains, the functional mechanisms of AI-2 have been well studied; however, in clinical antibiotic-resistant *E. coli* strains, whether AI-2 affects the expression of antibiotic resistance genes has not been reported. In this study, we report that exogenous AI-2 increased the antibiotic resistance of a clinical *E. coli* strain isolated from a dairy cow with mastitis by upregulating the expression of TEM-type enzyme in an LsrR (LuxS regulated repressor)-dependent manner. **Key words:** *Escherichia coli*, mastitis, autoinducer 2 (AI-2) quorum sensing, extended spectrum β -lactamase

Short Communication

Quorum sensing (QS) is a process in which bacteria use chemical molecules as a signaling language to change behaviors to adapt to specific environments (Fuqua et al., 1994; Bassler, 1999, 2002; Miller and Bassler, 2001). It has been reported that QS bacteria with high density in culture exhibit decreased susceptibility to antibiotics, reducing the responsiveness to antibiotics (Liu et al., 2000; Spoering and Lewis, 2001; Roberts and Stewart, 2005). Autoinducer 2 (AI-2), which is produced by many species of gram-negative and gram-positive bacteria, is thought to be a QS signaling molecule involved in interspecies communication (Federle and Bassler, 2003; Sun et al., 2004; Vendeville et al., 2005; De Keersmaecker et al., 2006). Autoinducer 2 signals are a set

of related molecules that are derived from spontaneous rearrangement of dihydroxy-2,3-pentanedione (DPD; Winzer et al., 2002; Miller et al., 2004). It has been reported that AI-2 QS affects antibiotic susceptibility in *Streptococcus anginosus* and *Staphylococcus aureus* (Ahmed et al., 2007; Xue et al., 2013). In *Escherichia coli* model strains, the functional mechanisms of AI-2 have been well studied (Wang et al., 2005; Xue et al., 2009); however, these model strains generally do not contain antibiotic resistance genes. Whether AI-2 affects the expression of antibiotic resistance genes in clinical antibiotic-resistant *E. coli* strains has not been reported.

The β -lactam groups of antibiotics such as penicillin or ampicillin have been the drugs of choice for treatment of bacterial infections. However, the emergence of the extended spectrum β -lactamase (ESBL) resistance mechanism brought a great challenge for the common use of β -lactam antibiotics and routine antimicrobial therapy (Jacoby and Medeiros, 1991; Bush, 2001; Altayb et al., 2014). In this study, strain ECDCM1 was a clinical *E. coli* strain isolated from a dairy cow that suffered from mastitis. The MIC assays showed that strain ECDCM1 was resistant to several β -lactam antibiotics, including ampicillin, oxacillin, and penicillin. The TEM-type ESBL are recognized as one of the most prevalent mechanisms of resistance to β -lactam antibiotics in the *Enterobacteriaceae* (Bonnet et al., 1999; Pai et al., 2001). The multi-antibiotic resistance of strain ECDCM1 is likely related to TEM. Thus, the genomic DNA of strain ECDCM1 was extracted and the TEM gene was detected by PCR. As expected, the TEM gene was amplified successfully and the fragments were sequenced. According to the sequencing results, strain ECDCM1 contains the typical TEM-type gene TEM-1.

Growth assays were performed according to the following methods. Colonies of strain ECDCM1 were placed into 2 mL of Mueller-Hinton (MH) broth (Oxoid, Basingstoke, UK) and cultivated at 37°C with shaking at 200 rpm for 16 h. Then, the overnight cultures were inoculated into 20 mL of fresh MH medium and diluted

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Table 1. Oligonucleotide primers (F = forward; R = reverse) used in this study

Primer name	Oligonucleotide (5'-3')
rt-16s-f	F: TTTGAGTTCCCGGCC
rt-16s-r	R: CGGCCGCAAGGTAA
rt-TEM-f	F: AGAGTTTTCGCCCCG
rt-TEM-r	R: CCCCCATGTTGTGCA
rt-lsrR-f	F: CGGATCGCGTGGTTTT
rt-lsrR-r	R: TCAACATATGCGCCGC
g-TEM-f	F: ATGAGTATTCAACATTTTCG
g-TEM-r	R: TTACCAATGCTTAATCAGTG

to a final concentration of OD₆₀₀ (optical density at 600 nm) 0.05 and, when necessary, ampicillin, oxacillin, or penicillin was added. The cell density was detected at each time point by using a UV/Vis spectrophotometer (DU730; Beckman Coulter, Miami, FL).

Antibacterial assays were performed according to a previous study (Chen et al., 2015). The cells of strain ECDCM1 were first exposed to antibiotics with or without exogenous AI-2 for 12 h at 37°C and were then inoculated into fresh MH broth and spread onto MH agar plates. After culturing for 24 h at 37°C, colony-

Figure 1. The growth curves (measured as optical density at 600 nm, OD₆₀₀) of *Escherichia coli* strain ECDCM1 and strain ECDCM1 cultured with 39 μM autoinducer 2 (AI-2) under different antibiotic conditions: (A) without any antibiotics, (B) with 6 mg/mL ampicillin, (C) with 3 mg/mL oxacillin, and (D) with 1 mg/mL penicillin G. Error bars indicate standard deviations. The results represent a mean of 3 independent experiments; **P* < 0.05, indicating a difference between antibiotic alone and antibiotic + AI-2.

forming units were counted and viable colonies between the control and test group were compared. The microbial counts of the control group exposed to antibiotics only (no AI-2) were designated as 100%.

The transcript levels of genes associated with antibiotic resistance and AI-2 were tested by performing real-time reverse transcription (RT)-PCR experiments. Cells were collected and resuspended in Tris-EDTA buffer (pH 8.0) containing 10 g/L lysozyme. After incubation for 5 min at 37°C, total RNA was extracted from cells using the Trizol reagent (Ambion, Austin, TX), and residual DNA was removed using DNaseI (TaKaRa, Dalian, China). Real-time RT-PCR was performed using the PrimeScript 1st Strand cDNA synthesis kit, SYBR Premix Ex Taq (TaKaRa), and a StepOne real-time PCR system (Applied Biosystems, Carlsbad, CA). Difference of gene expression was calculated by $\Delta\Delta C_t$ (where C_t = cycle threshold) method, using 16S gene as the housekeeping gene, normalized by subtracting the C_t value of 16S cDNA from target cDNA. All primers used in this study are listed in Table 1 and had PCR efficiency between 1.93 and 2.09.

The digoxigenin (DIG) gel shift assays were performed according to a previous study (Xue et al., 2009). The transcriptional regulator LsrR protein was overexpressed and purified according to the methods described previously (Xue et al., 2009) and the promoter region of *TEM* was amplified by PCR. The PCR products were labeled using the DIG gel shift kit (Roche, Indianapolis, IN) and were incubated at 25°C for 15 min with various amounts of LsrR protein. After incubation, the mixtures were electrophoresed in a 4.5% native polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. The band shifts were detected by the DIG gel shift kit.

All data were analyzed using the *t*-test ANOVA, with a *P*-value <0.05 considered significant. The experiments were repeated at least 3 times with similar results, and 1 representative result is shown.

To determine the effect of AI-2 on survival of strain ECDCM1 in the presence of antibiotics (ampicillin, oxacillin, and penicillin), cells of strain ECDCM1 were cultured in MH broth, and growth curves were detected under different conditions. The growth of strain ECDCM1 was first observed with the addition of exogenous AI-2 only, without any kind of antibiotics. The growth curve of strain ECDCM1 was similar to that of a test group cultured with exogenous AI-2 at 39 μ M (Figure 1A). Subsequently, we evaluated the effect of AI-2 in the presence of antibiotics. Ampicillin, oxacillin, and penicillin were added to the culture medium at concentrations of 6, 3, and 1 mg/mL, respectively. As shown in Figure 1B and Figure 1D, in the presence of ampicillin or penicillin, addition of exogenous AI-2

Figure 2. Colony-forming units assays of *Escherichia coli* strain ECDCM1 and strain ECDCM1 cultured with 39 μ M autoinducer 2 (AI-2) under different antibiotic conditions. The survival rate of the control group without addition of AI-2 was designated 100%. The colony counts of the test group cultured with AI-2 under different antibiotics (A = ampicillin, B = oxacillin, C = penicillin G) were compared with that of the control group (without AI-2). Error bars indicate standard deviations. The results represent a mean of 3 independent experiments; ***P* < 0.01, **P* < 0.05, indicating a difference between antibiotic alone and antibiotic + AI-2.

Figure 3. Comparative measurement of transcription (cDNA abundance) of *TEM* and *lsrR* in *Escherichia coli* strain ECDCM1. Relative transcript levels of *TEM* and *lsrR* were tested by real-time reverse transcription-PCR in strain ECDCM1 and strain ECDCM1 cultured with 39 μ M autoinducer 2 (AI-2) under different antibiotic conditions: (A) without any antibiotics, (B) with 6 mg/mL ampicillin, (C) with 3 mg/mL oxacillin, (D) with 1 mg/mL penicillin G. Error bars indicate standard deviations. The results represent a mean of 3 independent experiments; ** $P < 0.01$, indicating a difference between antibiotic alone and antibiotic + AI-2.

led to an increase of cell density of strain ECDCM1 at each culture time point. Moreover, in the presence of oxacillin, addition of AI-2 not only increased the cell density of the bacteria, but also increased the growth rate of the cells at h 10 and 12 (Figure 1C). These results indicated that the antibiotic resistance of this strain increased upon addition of AI-2.

To further examine changes in the antibiotic resistance of strain with addition of exogenous AI-2, antibacterial assays were performed. The survival rates of the test groups with exposure to antibiotics and AI-2 were higher than that of the control group (Figure 2). The survival rate of the test group with oxacillin (5 mg/mL) and AI-2 was almost 4 times higher than that

of the control group (Figure 2B). These results indicate that with addition of exogenous AI-2, resistance of the strain ECDCM1 to all 3 antibiotics increased, suggesting that this clinical *E. coli* isolate might use AI-2 as a signal to upregulate its antibiotic resistance.

To investigate the mechanism of how AI-2 affects resistance to antibiotics in *E. coli*, the transcript levels of genes associated with antibiotic resistance and AI-2 were assessed by performing real-time RT-PCR experiments. The *TEM* gene is important in β -lactam antibiotic resistance and is related to resistance to ampicillin, oxacillin, and penicillin in *E. coli* (Jacoby and Medeiros, 1991; Pai et al., 2001). The transcript levels of *TEM* and *lsrR* were measured with or without ad-

Figure 4. The binding ability of LsrR to the *TEM* promoter was determined by gel-shift assays. Increasing amounts of LsrR were incubated with digoxigenin (DIG)-labeled *TEM* promoters (DIG-p-TEM). In each panel, from lanes 1 to 6, the amounts of LsrR were 4, 0, 0.5, 1, 2, and 4 pmol, respectively; 50 fmol of DIG-labeled probe was added to all lanes. In lane 1, except for the labeled probes, 1 pmol of unlabeled probe was added as the competitive control (Ctrl).

dition of exogenous AI-2 and with different antibiotics. As shown in Figure 3A, the transcription of *TEM* was not apparently affected by the addition of exogenous AI-2 without the presence of antibiotics. However, as shown in Figure 3B, C, and D, in the presence of antibiotics, addition of AI-2 resulted in 2.1-fold (ampicillin), 2.4-fold (oxacillin), and 2.4-fold (penicillin) increases in transcription of *TEM*, suggesting that exogenous AI-2 might upregulate the antibiotic resistance of strain ECDCM1 through *TEM*. Moreover, the transcript level of *lsrR*, which encodes the receptor of AI-2, was measured. Our results showed that in the presence of antibiotics, the exogenous AI-2 led to 3.8-fold (ampicillin), 3.7-fold (oxacillin), and 13.6-fold (penicillin) increases in *lsrR* transcription in strain ECDCM1, further supporting that, in ESBL-positive *E. coli*, AI-2 might regulate the antibiotic resistance gene *TEM* in an LsrR-dependent manner.

Because LsrR contains a helix-turn-helix (HTH) DNA binding domain and has been reported as a QS regulator that can regulate the transcription of a variety of genes by binding to their promoters, we hypothesize that LsrR regulates *TEM* transcription by binding directly to its promoter region. To determine whether LsrR can bind to the promoter of *TEM*, we performed DIG-gel shift assays. As shown in Figure 4, LsrR exhibited strong binding ability to the promoter of *TEM*, confirming that LsrR regulates *TEM* transcription by binding directly to its promoter region.

In conclusion, AI-2 can regulate the expression of *TEM* through the AI-2 receptor LsrR. Moreover, in the presence of antibiotics, the antibiotic-resistant *E. coli* strain exhibited stronger antibiotic resistance in response to an increase in cell density. These data indicate that long-term exposure to antibiotics may force this strain to evolve a better survival strategy.

The AI-2 QS system may be a very important system for adaptation to a changing environment in a range of gram-negative and gram-positive bacteria. Although the TEM-type ESBL are among the most common mechanisms for antibiotic resistance in *E. coli*, which genes can regulate *TEM* has seldom been reported. This study provides new clues to the *TEM*-regulating mechanism and suggests that a treatment other than antimicrobial treatment should be considered in ESBL-positive *E. coli*.

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