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Master's Thesis of Science
In Agricultural Biotechnology

Effects of the acquisition of *mcr-1*-harboring plasmids on virulence in enterohemorrhagic *Escherichia coli*

mcr-1 보유 플라스미드의 획득에 의한
장출혈성 대장균의 병원성에 대한 영향 분석

August 2021

The Graduate School
Seoul National University
Department of Agricultural Biotechnology

Eunbyeol Ahn

Effects of the acquisition of *mcr-1*-harboring plasmids on virulence in enterohemorrhagic *Escherichia coli*

Advisor: Sangryeol Ryu

Submitting a master's thesis of
Science in Agricultural Biotechnology

August 2021

The Graduate School
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Abstract

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Colistin is considered as a last resort antibiotic against multidrug-resistant Gram-negative bacteria. However, recently, the mobilized colistin resistance (*mcr*) *-1* gene was first discovered in *Escherichia coli* in China. Since then, dissemination of the *mcr-1* by plasmids in pathogenic *Escherichia coli* including enterohemorrhagic *E. coli* (EHEC) is a concern to global public health. In Korea, the *mcr-1*-harboring plasmids were isolated from food, human, and livestock. The whole-genome sequencing analysis revealed that *mcr-1*-harboing plasmids encode a number of virulence-related genes including pili and type IV secretion systems. In this study, I investigated the effects of *mcr-1*-harboring plasmid on the virulence of EHEC. The *mcr-1*-harboring plasmid was easily transferred to EHEC ATCC 43894 by conjugation and remained stable in ATCC

43894 even after 10 consecutive subcultures. Based on the pan-genome analysis of IncI2 type *mcr-1*-harboring plasmids mainly discovered in Asia, I selected three genes which are related to virulence (*pilS* and *virB2*) including *mcr-1* and constructed their mutant strains for characterization of various virulence-associated traits. As a results, *pilS* and *virB2* did not affect the virulence of ATCC 43894. Although the acquisition of the *mcr-1* did not affect the growth of ATCC 43894, it decreased the swimming motility. Also, the *mcr-1* decreased cell surface hydrophobicity of ATCC 43894 resulting in the reduction of biofilm formation. The expression of the *mcr-1* increased bacterial adherence to human epithelial HEp-2 cells, while it decreased invasiveness of ATCC 43894. Interestingly, band patterns of core-lipid A and O-antigen region analyzed by deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE) were altered by the *mcr-1*, suggesting that the *mcr-1* modified the polysaccharide as well as lipid A. Collectively, the results in this study demonstrate that the acquisition of *mcr-1* not only conferred colistin resistance but also can affect virulence in ATCC 43894.

Keywords: *mcr-1*, enterohemorrhagic *Escherichia coli*, antibiotic resistance

Student Number: 2019-20901

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1. Introduction

Colistin (Polymyxin E) is considered the last resort for the treatment of multidrug-resistant Gram-negative bacteria. The colistin resistance mechanisms have mainly reported chromosomally mediated that mutations in two-component regulatory systems PmrAB and PhoPQ (Poirel, Jayol et al. 2017). But, the mobilized colistin resistance gene, *mcr-1*, was first described in *Escherichia coli* in China, 2015 (Liu, Wang et al. 2016). Since then, the discovery of *E. coli* harboring *mcr-1* has been reported numerous countries including America, Asia, and Europe ((Liu, Wang et al. 2016), (Monte, Fernandes et al. 2017), (El Garch, Sauget et al. 2017)) and isolated from various sources such as food, livestock, and human (Irrgang, Roschanski et al. 2016), (Liu, Wang et al. 2016), (Veldman, van Essen-Zandbergen et al. 2016). In Korea, *mcr-1*-harboring plasmids in *E. coli* strains have been also reported (Lim, Kang et al. 2016), (Yoon, Hong et al. 2018), (Kim, Hwang et al. 2019). The dissemination of the *mcr-1* by plasmids causes serious clinical

problems in many countries.

Lipopolysaccharide (LPS) is a main components of the outer membrane in most Gram-negative bacteria. LPS provides the structural integrity of outer membrane and interacts with a surface between the bacterium and its environment (Nakao, Ramstedt et al. 2012). LPS structures consist of three components: the lipid A, which is the hydrophobic portion of the molecule anchored in the outer membrane; the O-antigen extending from the cell to the external environment; and the core oligosaccharide, which links the O-antigen to the lipid A. LPS triggers the release of many inflammatory cytokines, and it has been implicated as the etiological agent of a variety of pathologies (Nakao, Ramstedt et al. 2012).

Colistin is amphipathic antibiotic with hydrophobic fatty acyl moiety and hydrophilic L-diaminobutyric acid amino groups. The binding of colistin to the bacterial surface depends on the electrostatic interaction between the negatively charged

phosphate group of lipid A of LPS and the positively charged colistin (Gao, Hu et al. 2016). However, *mcr-1* encodes a phosphoethanolamine (PEtN) transferase that adds PEtN to lipid A of LPS. The LPS modification mediated by *mcr-1* results in neutralize the negative chare of LPS and subsequently reduces binding affinity to colistin (Gao, Hu et al. 2016).

Enterohemorrhagic *Escherichia coli* (EHEC) is a food-borne pathogen of worldwide importance that causes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Besser, Griffin et al. 1999). This pathogen has caused a number of significant outbreaks of foodborne disease worldwide. EHEC forms attaching and effacing (AE) lesions on human epithelial cells and produces Shiga-like toxins. EHEC is also able to form biofilms and these biofilms are resistant to conventional antimicrobial agents (Kim, Lee et al. 2016). Recently, *mcr-1* was detected in several EHEC strains (Lopes, Costa et al. 2020), (Palupi, Wibawan et al. 2019). The presence of the *mcr-1* in EHEC is of concern because it is pathogenic to humans, posing a public health problem.

Previous study reported that *mcr-1*-harboring plasmids are easily

transferred by conjugation in pathogenic *E. coli* strains including EHEC (Kim, Hwang et al. 2019). Also, the whole-genome sequencing analysis revealed that *mcr-1*-harboring plasmids encode a number of virulence-related genes, such as those encoding pili and type VI secretion systems. Nevertheless, researches on *mcr-1* are limited to antibiotic resistance studies, and research on virulence of *mcr-1* is still insufficient. Thus, in this study, I investigated that the horizontal transfer of *mcr-1*-harboring plasmids can affect virulence of ATCC 43894.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

E. *coli* ATCC 43894 (Enterohemorrhagic *E. coli*; EHEC) was cultured on Luria-Bertani (LB) media (Difco, USA) at 37℃. The *mcr-1*-harboring plasmid used in this study was found in FORC82 isolated from retail raw chicken in South Korea (Kim, Hwang et al. 2019). Antibiotics and chemicals were supplemented to the media at the final concentrations of 2 μg/ml (colistin), 50 μg/ml (ampicillin) and 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

2.2 Construction of the knockout mutants

The primers used in this study are shown in Table 1. The *mcr-1*-harboring plasmid used in this study was isolated retail raw chicken in Korea (Kim, Hwang et al. 2019). The *mcr-1*-harboring plasmid in FORC82 was introduced into the ATCC 43894 by conjugation. The three mutants (*mcr-1*, *pilS*, *and virB2*) were constructed using a

one-step inactivation method (Datsenko and Wanner 2000).

2.3 Plasmid stability test

The plasmid stability test was performed as described previously (Nang, Morris et al. 2018). Briefly, transconjugant were grown overnight in LB broth with colistin. 10 μl of overnight seed cultures were inoculated in 10 mL fresh LB broth without antibiotic (1:1000 ratio). Subculturing was performed after 24 h incubation and repeated for 10 consecutive days. For each culture, culture solution was serially diluted, spread on LB agar plates, and incubated at 37℃ overnight. Identification of *mcr-1*-loss cells within each culture was determined by transferring 96 colonies from LB agar plate to 4 μg/mL colistin-containing LB broth in each well of a 96-well plate. The proportion of plasmid-containing cells were calculated by counting the number of wells that had visible growth of bacteria. FORC82 was used as the control strain.

2.4 Pan-genome analysis

Twenty of IncI2 type *mcr-1*-harboring plasmids were identified with GView Pangenome analysis tool.

2.5 Biofilm formation assay

A static biofilm formation assay was performed as described previously (Kim, Lee et al. 2016). Briefly, overnight cultures were inoculated in LB broth (total volume 200 μl) at an initial turbidity of 0.05 at 600 nm and cultured in 96-well polystyrene plates for 72 h without shaking at 37℃. To quantify biofilm formation, cell cultures were washed three times with PBS to remove all non-adherent cells. Biofilms were stained with 0.1% crystal violet for 20 min, rinsed three times with PBS, extracted with 33% acetic acid, and absorbances were measured at 570 nm.

2.6 Swimming motility assay

Swimming motility assay was performed at 37℃ on 0.3% agar plates

containing 1% tryptone and 0.25% NaCl. The motility halos were measured after a 30 h incubation.

2.7 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). Minimum inhibitory concentration (MIC) of colistin was determined according to CLSI guidelines. *E. coli* ATCC 25922 was used as the quality control.

2.8 Growth curves

Growth curves were determined by measuring the optical density at 600 nm. Growth rates was measured at 37℃ for 24 h.

2.9 Cell surface hydrophobicity test

The cell surface hydrophobicity was measured as described

previously (Arkoun, Daigle et al. 2017). In brief, a 10 mL LB broth was inoculated with 100 μL from an overnight culture. The suspension was then incubated at 37℃ and allowed to grow up to an optical density of 0.5. Thereafter, 4 mL of this suspension was transferred into a 15 mL polypropylene tube. A first measurement of optical density OD_{600} was then carried out and recorded as Ab_{st0}. 500 μL of hexane were added to the suspension and the mixture was vortexed for 1 min and then allowed to rest for 15 min at room temperature to make phase separation. A second OD_{600} measurement of aqueous phase was recorded as Ab_{st1}. Finally, the cell hydrophobicity was calculated according to Equation.

$$\%\ Hydrophobiciy = \frac{Abst_0 - Abst_1}{Abst_0} \times 100$$

2.10 Bacterial adhesion and invasion assay

Adhesion and invasion assays were performed as described previously (Andreozzi, Gunther IV et al. 2018). Briefly, HEp−2, human laryngeal epithelial cells, were cultured in Eagle's minimal

essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) at 37℃ under 5% CO_2. A monolayer of 4×10^5 HEp-2 cells was prepared in a 24-well tissue culture plate. 150 μl overnight cultures were inoculated in 3 ml LB broth and incubated at 37℃ at 220 rpm until the OD_{600} was 1. The bacterial suspension was diluted in EMEM with 10% FBS, and then added onto the cell monolayer at a multiplicity of infection (MOI) of 10. For invasion assays, bacteria were allowed to adhere to HEp-2 cells for 3 h and treated with 100 μg/ml gentamicin for 2 h to kill extracellular bacteria. The wells were washed three times with pre-warmed PBS and lysed in 1% Triton X-100 for 15 min, and then serially diluted in PBS. A dilution of the suspension was plated on LB agar plates to enumerate the CFU. All adhesion/invasion assays were done in triplicate wells.

2.11 LPS extraction and analysis

The LPS extraction was performed as described previously (Kim and Ryu 2012). Briefly, LPS was extracted from overnight bacterial cultures using hot phenol-water micro-extraction methods. The

bacteria suspension (2×10^9 CFU/mL) was harvested, washed once with 1 ml DPBS (Dulbecco' s PBS containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$), and centrifuged. Pellets were resuspended in 100 μl DPBS and sonicated. In order to eliminate contaminating protein and nucleic acids, proteinase K (100 μg/mL) was added and incubated at 37℃ for 1 h. Then 200 μl of ddH_2O and 300 μl of pre-heated (68℃) phenol solution were added prior to incubation at 68℃ with vigorous vortex mixing every 5 min. The samples were chilled on ice for 5 min, and the aqueous phases were separated by centrifugation at 10,000 x g, 4℃ for 5 min. The LPS was extracted again from the phenol phase with another 300 μl of ddH_2O. After the addition of sodium acetate to the pooled aqueous phases at a final concentration of 0.5 M, 10 ml of 95% ethanol was added, and the mixture was incubated overnight at −20℃. The crude LPS were sedimented by centrifugation at 10,000 × g, 4℃ for 5 min, re-suspended in 100 μl ddH_2O, and precipitated with 95% ethanol. Finally, the precipitated LPS was re-dissolved in 50 μl ddH_2O and stored at −20℃. The extracted LPS were analyzed

by DOC-PAGE on a 15% acrylamide gels. The gels were pre-run with a running buffer (290 mM glycine, 37 mM Tris base and 6 mM sodium deoxycholate) for 10 min at 15 mA using Bio Rad Mini-PROTEAN® Tetra Cell. The extracted LPS was mixed with equal volumes of sample buffer (containing 2 ml of stacking gel buffer solution, 1 ml of glycerol and 2.5 mg of bromophenol blue, brought up to 10 ml with ddH_2O), and the mixtures were loaded onto the gels prior to running each gel for ~ 60 min at 15 mA. The gels were fluorescently stained using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Molecular Probes, Cat. No. P20495; Eugene, OR, USA) according to the manufacturer's instructions. The samples were visualized under the 300 nm UV by the Red™ Imaging System.

2.12 Statistical analysis

The statistical analysis was conducted using the GraphPad Prism (GraphPad Software, Inc., USA, Version 5.01). All results were analyzed by student's t test.

Table 1. Primers used for the construction of bacterial strains and plasmids

Primer	Sequences (5' to 3')[a]
mcr-1-P1-Kan-F	ATGGAGTGTGCGGTGGGTTTGGAAAAAATACAGGGAGAAA TGTAGGCTGGAGCTGCTTCG
mcr-1-P4-Kan-R	GCCGCAATTATCCCACCGTTTATTTTTGAGTAGTTTCTC CTG TCA AAC ATG AGA ATT AAT TCC
pilS-P1-Cm-F	TGCACGCCGTAAAAAAGAACAGGACAAAGGGGCAACACTG GTGTAGGCTGGAGCTGCTTC
pilS-P2-Cm-R	AATAAGCGCCCGAAGGCGCTTATCCGATGCACATGAAAAA ATG GGA ATT AGC CAT GGT CC
virB2-P1-Cm-F	AAACATCACCGGACTGGGATGTTTTTGGAGATTTTAATTA GTGTAGGCTGGAGCTGCTTC
virB2-P2-Cm-R	CAT AAG GTA AAT ATG CGG CAC AGT TGT ATA TAT GGA TTA C ATG GGA ATT AGC CAT GGT CC
mcr-1-con-F	CATCTCAGCAAGTAGGCGTT
mcr-1-con-R	GAA TTG CCG CAA TTA TCC CAC
pilS-con-F	GTC AAA TCA ACA GGA GTC TGG
pilS-con-R	CAC AAT ACT CCT CCT TAA ACA TG
virB2-con-F	CGT GCC ATC AAT CCG TGA AA
virB2-con-R	CTC ACC ATG AAA AAA GCC GC

Kan-con-F	GCA AGG TGA GAT GAC AGG AG
Kan-con-R	CGA CCA CCA AGC GAA ACA TC
Cm-con-F	CACGCCACATCTTGCGAATA
Cm-con-R	GAA TAT GTT TTT CGT CTC AGC C
SalI-mcr-1-F	TTGTC GAC ATG ATG CAG CAT ACT TCT GTG T
EcoRI-mcr-1-R	AAGAATTC GGG AGA AAT CAG CGG ATG AA

[a] Restriction enzyme sites are underlined.

3. Results

3.1 Plasmid stability

A plasmid stability test was conducted to investigate how stable the *mcr-1*-harboring plasmid transferred by conjugation was maintained in the recipient strain. FORC82 was used as the control strain. As a result, *mcr-1*-harboring plasmid in ATCC 43894 remained stable. No plasmid loss was found in both strains for 10 consecutive subcultures (Fig. 1).

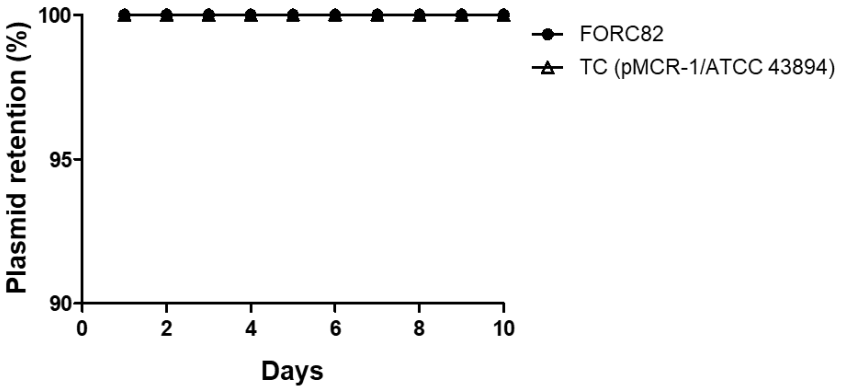


Fig. 1. Stability of the *mcr-1*-harboring plasmid in ATCC 43894.

Subculture was repeated for 10 consecutive days in the absence of colistin. FORC82, which originally has *mcr-1*-harboring plasmid, was used as the control strain.

3.2 Pan-genome analysis

Based on the pan-genome analysis of IncI2 type *mcr-1*-harboring plasmids mainly discovered in Asia including Korea, I selected three genes (*mcr-1*, *pilS*, and *virB2*) and constructed their mutant strains for characterization of various virulence-associated traits.

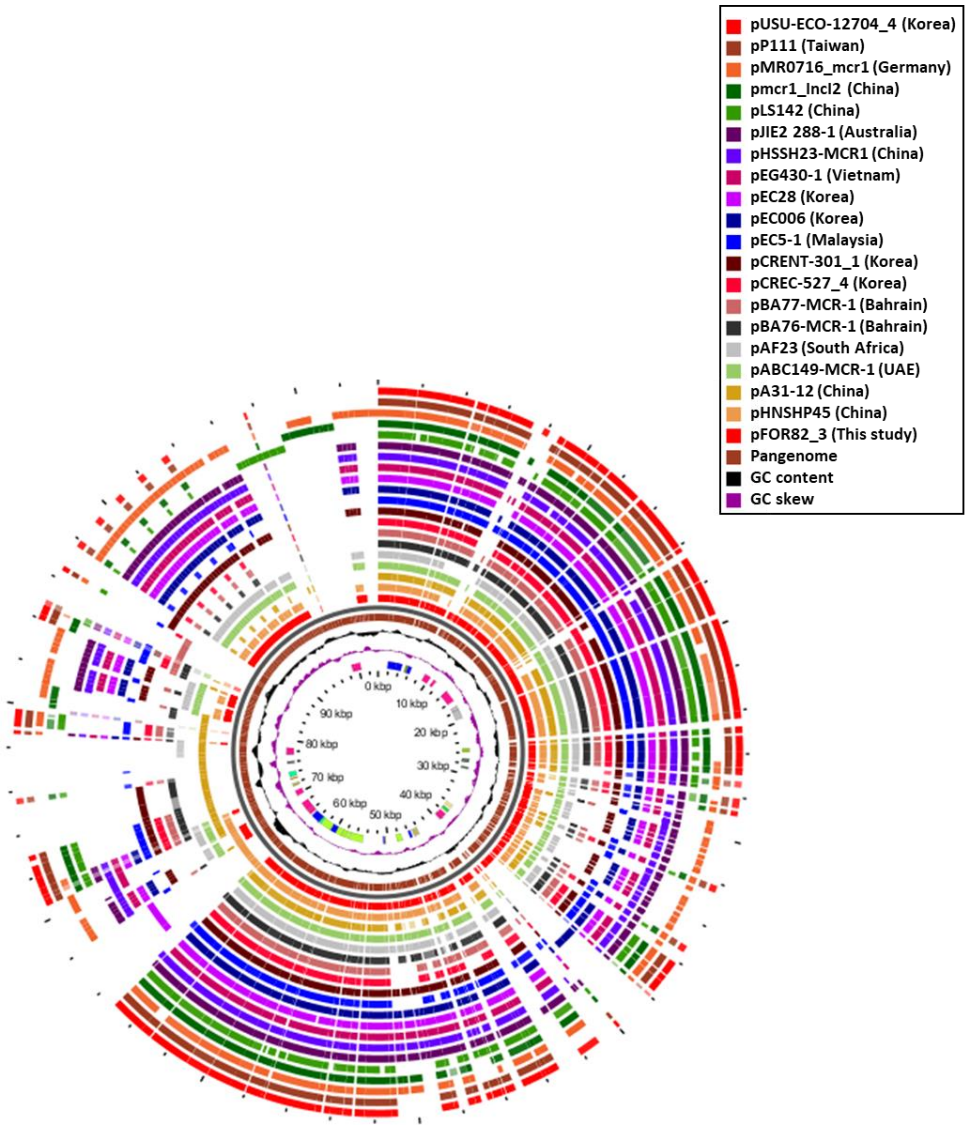
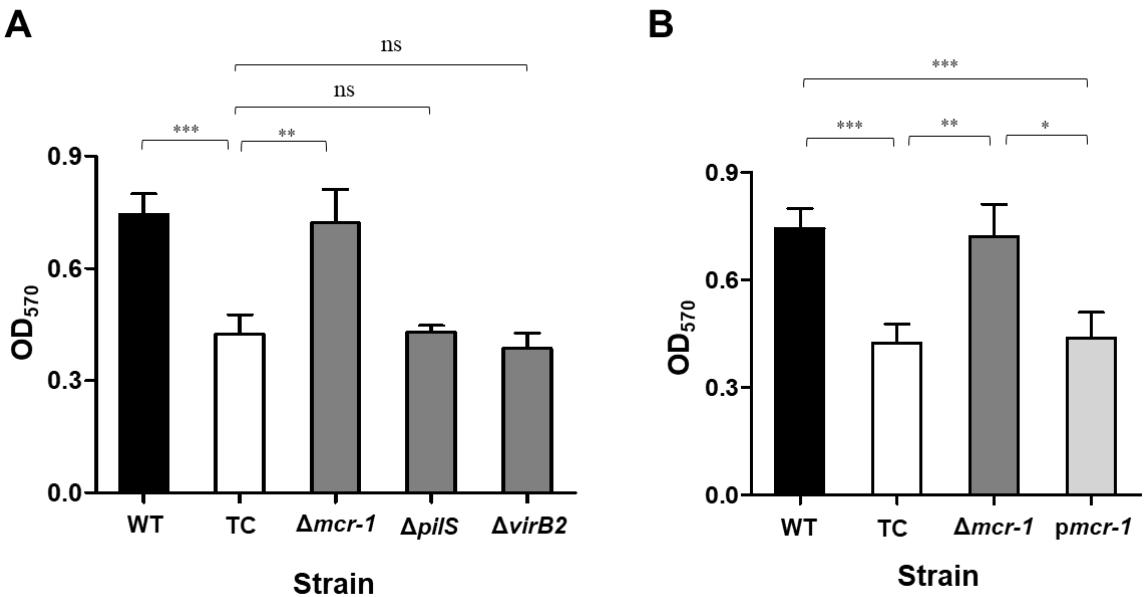


Fig. 2. Pan-genome analysis of *mcr-1*-harboring plasmids.

3.3 Biofilm formation

As biofilm is important virulence factor for pathogenic *E. coli*, I compared to biofilm formation between wild type and the transconjugant. Transconjugant was noticeably defective in biofilm compared to that of wild type. In order to identify which genes on *mcr-1*-harboring plasmids affect the ability of biofilm formation, I measured the biofilm of the three mutant strains (Fig. 3A). Especially, the biofilm formation of *mcr-1* mutant strain restored to the wild type levels. In contrast, both *pilS and virB2* mutant strains retained their ability to form biofilm, similar to the transconjugant. To confirm that the *mcr-1* was responsible for the reduced capacity to form a biofilm, the pUC19::*mcr-1* was constructed and introduced into the *mcr-1* mutant strain. The ability to form biofilm of *mcr-1* complemented strain was reduced to transconjugant levels (Fig. 3B). These results suggested that the acquisition of *mcr-1* had an effect on the reduction of biofilm formation capacity of ATCC 43894.


A
ns
ns
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**
0.9
0.6
0.3
0.0
OD570
WT
TC
Δmcr-1
ΔpilS
ΔvirB2
Strain
B
***
***
**
*
0.9
0.6
0.3
0.0
OD570
WT
TC
Δmcr-1
pmcr-1
Strain

Fig. 3. Biofilm formation.

(A) Biofilm formation in wild type, transconjugant, and the three mutants (Δ*mcr-1*, Δ*pilS,* and Δ*virB2*)

(B) Biofilm formation in wild type, transconjugant, *mcr-1* mutant, and *mcr-1* complemented strain. Biofilms were grown in 96-well plates at 37℃ for 72 h. The biofilms were visualized and quantified by crystal violet staining. Statistical significance was evaluated with Student's t test using GraphPad Prism. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

3.4 Swimming motility

I also measured swimming motility that one of the virulence factors of pathogenic *E. coli*. The tranconjugant was defective for swimming motility compared to that of wild type. Also, swimming motility restored in *mcr-1* mutant strain. The *pilS* and *virB2* mutant strains appeared to show similar swimming motility ability of transconjugant (Fig. 5A). In order to confirm that the *mcr-1* affect swimming motility, the pUC19::*mcr-1* was introduced into *mcr-1* mutant strain. Swimming motility of the *mcr-1* complemented strain was decreased compared to the *mcr-1* mutant strain (Fig. 5B). These results indicate that *mcr-1* affect the reduction of swimming motility ability of ATCC 43894.

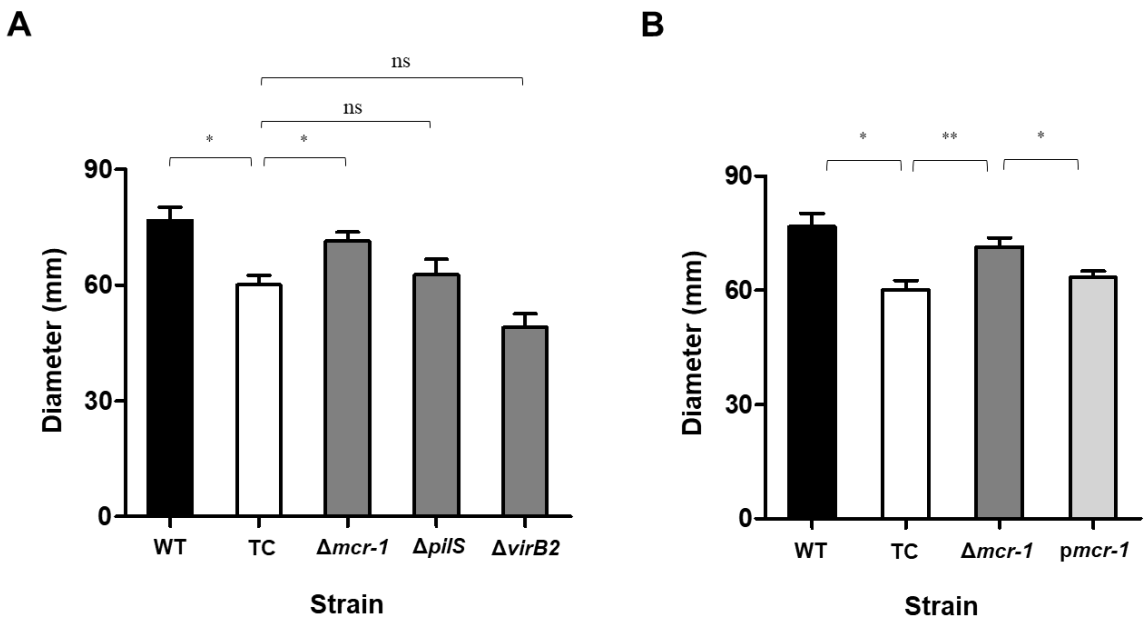

A
ns
ns
*
*
90
60
30
0
Diameter (mm)
WT
TC
Δmcr-1
ΔpilS
ΔvirB2
Strain
B
*
**
*
90
60
30
0
Diameter (mm)
WT
TC
Δmcr-1
pmcr-1
Strain

Fig. 4. Reduced swimming motility by the acquisition of *mcr-1*.

(A) Swimming motility of wild type, transconjugant, and the three mutants (Δ*mcr-1*, Δ*pilS,* and Δ*virB2*)

(B) Swimming motility of wild type, transconjugant, *mcr-1* mutant, and *mcr-1* complemented strains. Statistical significance was evaluated with Student ' t test using GraphPad Prism. *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

3.5 Antimicrobial susceptibility testing

To evaluate the effects of *mcr-1* on colistin resistance and confirm the construction of the *mcr-1* mutant and *mcr-1* complemented strains, MIC test was conducted with colistin. The MIC of transconjugant

Table 2. Minimum inhibitory concentration (MIC) of colistin

Strain	*mcr-1* gene [a]	MIC[b] (μg/mL)
		COL
WT	-	4
TC	+	8
Δ*mcr-1*	-	4
p*mcr-1*	+	8

[a] Presence (+) or absence (-) of *mcr-1*, based on PCR and confirmed by sequencing. [b] COL: colistin.

3.6 Growth curve

A growth curve was performed to investigate the *mcr-1* affects fitness burden on bacteria. The growth rates of transconjugant were not different compared to that of wild type. No significant differences in the growth rates were found between the strains (Fig. 5). These results suggest that *mcr-1* no effect on growth of ATCC 43894.

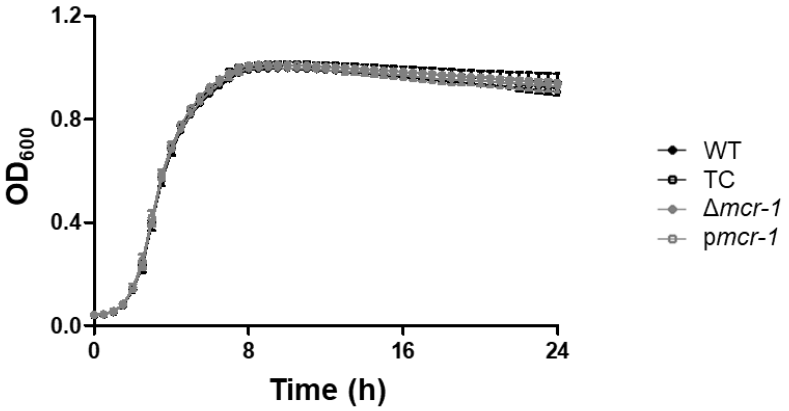


Fig. 5. No impact of the presence of the *mcr-1* on the growth of ATCC 43894.

Growth of wild type, transconjugant, *mcr-1* mutant, and *mcr-1* complemented strains were determined by OD_{600}.

3.7 Cell surface hydrophobicity test

To determine whether *mcr-1* led to alteration in the cell surface hydrophobicity, the hydrophobicity values were estimated based on the ability to bind with hexane. As a result, a significantly decreased hydrophobicity was observed in transconjugant when compared against that of wild type. The *mcr-1* mutant strain was restored the cell surface hydrophobicity. As expected, the cell surface of *mcr-1* complemented strain was decreased compared to the *mcr-1* mutant strain (Fig. 6). These results suggested that expression of *mcr-1* decreased cell surface hydrophobicity, which can affect the reduction of the biofilm formation of ATCC 43894.

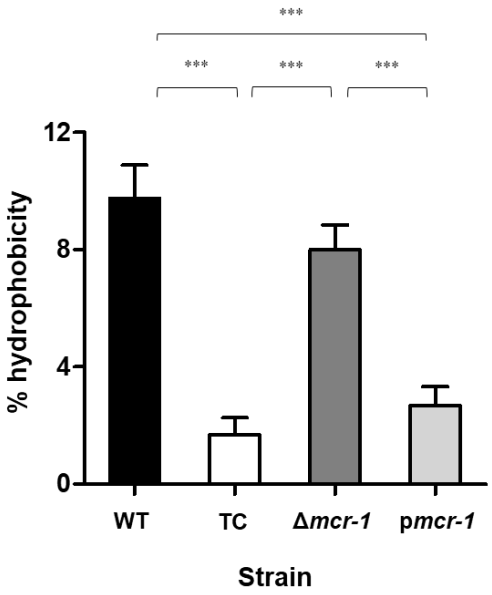
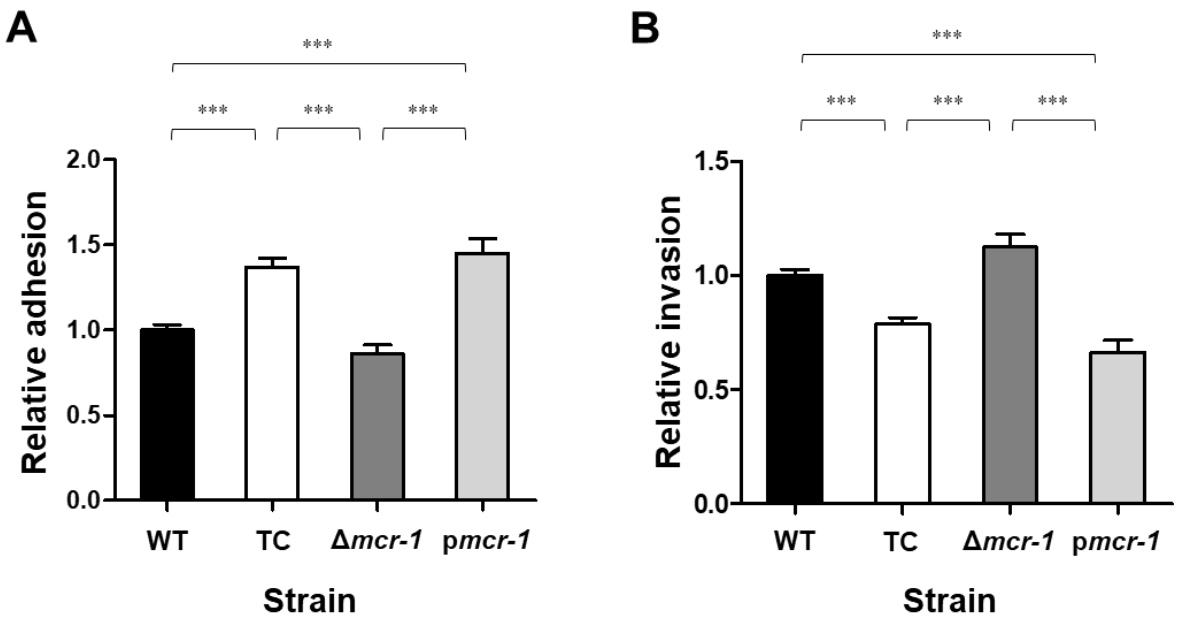


Fig. 6. Cell surface hydrophobicity.

Cell surface hydrophobicity was estimated by the bacterial adhesion to a hydrocarbon (BATH) method. Statistical significance was evaluated with Student ' s t test using GraphPad Prism. ***, $P < 0.001$. These experiments were repeated at least three times.

3.8 Bacterial adhesion/invasion assay

To determine that the *mcr-1* affect bacterial attachment to human epithelial cells, adhesion assay was performed to HEp-2 cells. The transconjugant exhibited significantly increased adherence ability compared to that of wild type strain, while the adherence of *mcr-1* mutant strain was restored. Compared with *mcr-1* mutant strain, *mcr-1* complemented strain displayed increase in adhesion to the HEp-2 cells (Fig. 5A). These results suggested that *mcr-1* may play a role in the colonization of ATCC 43894. I next investigated the effect of *mcr-1* in an invasion assay. The transconjugant had reduced invasion compared to that of wild type. As expected, ability of invasive to HEp-2 cells restored in *mcr-1* mutant strain. The invasive ability of *mcr-1* complemented strain decreased compared to *mcr-1* mutant strain (Fig. 5B). Based on these results, acquisition of *mcr-1* results in decreased invasiveness of ATCC 43894.


A
***
***
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Relative adhesion
2.0
1.5
1.0
0.5
0.0
WT
TC
Δmcr-1
pmcr-1
Strain
B
***
***
***
***
Relative invasion
1.5
1.0
0.5
0.0
WT
TC
Δmcr-1
pmcr-1
Strain

Fig. 7. Bacterial adhesion and invasion assay.

(A) Adhesion assay: Bacteria were inoculated onto monolayer of Hep-2 cells for 3 h. (B) Invasion assay: After adhesion, extracellular bacteria were killed by addition of gentamicin for 2 h. Statistical significance was evaluated with Student ' s t test using GraphPad Prism. ***, $P < 0.001$.

3.9 LPS extraction and analysis

To investigate *mcr-1* affected LPS structure modification, LPS of the wild type, transconjugant, *mcr-1* mutant, and *mcr-1* complemented strains were extracted and analyzed deoxychaolate-polyacrylamide gel electrophoresis (DOC-PAGE). Interestingly, the analysis of the LPS by DOC-PAGE revealed that transconjugant showed altered banding patterns for the core-lipid A and O antigen region compared to that of wild type. As expected, banding patterns were restored in *the mcr-1* mutant strains and appear to be the same as the wild type. In *mcr-1* complemented strains, the LPS profile was restored to the transconjugant (Fig. 7). These results indicated that expression of *mcr-1* modified the polysaccharide of LPS as well as lipid A.

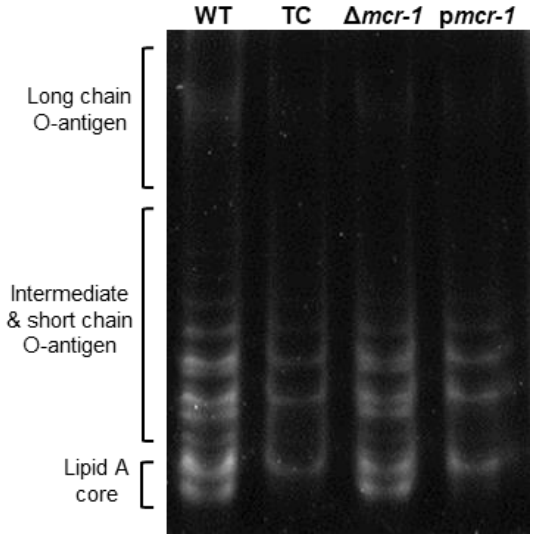

Fig. 8. DOC-PAGE analysis of LPS.

LPS of wild type, transconjugant, *mcr-1* mutant, and *mcr-1* complemented strains were extracted using hot phenol-water micro-extraction methods and analyzed by DOC-PAGE.

4. Discussion

In this study, I demonstrated that *mcr-1* not only conferred colistin resistance but also affect virulence of ATCC 43894. First, I identified that *mcr-1*-harboring plasmids transferred to ATCC 43894 by conjugation remained stable in ATCC 43894 for 10 consecutive subcultures without colistin. Previous study reported that *mcr-1*-harboring plasmids are easily transferred by conjugation among the bacteria in pathogenic *E. coli* (Kim, Hwang et al. 2019). The high stability of *mcr-1*-harboring plasmid in recipient strain and easy transfer to other bacteria may contribute to the wide spread of *mcr-1*.

Many experiments such as biofilm formation, swimming motility, and adhesion assay to HEp-2 cells were conducted to identify difference of virulence between wild type and transconjugant. Interestingly, there were significant difference between them. Thus, to investigate which genes of *mcr-1*-harboring plasmid affect the virulence of ATCC 43894 and compare identity of the genes between IncI2 type

mcr-1-harboring plasmids particularly discovered in Asia (Matamoros, Van Hattem et al. 2017), (Lim, Kang et al. 2016), (Kim, Chong et al. 2017), (Kim, Hwang et al. 2019), I conducted pan-genome analysis of 20 IncI2 type *mcr-1*-harboring plasmids. Based on the pan-genome analysis, I selected the three genes related to virulence (*pilS* and *virB2*) including *mcr-1*. PilS is a regulator of type VI pili and virB2 is a major pilus protein of the type VI secretion system (Boyd, Koga et al. 1994), (Backert, Fronzes et al. 2008). Then I constructed their mutant strains for characterization of virulence-related traits.

The result of biofilm formation assay exhibited that *mcr-1* had an effect on the reduction of biofilm formation. Several factors have been reported to affect biofilm formation such as cell surface hydrophobicity, surface charge, and surface structures (Van Houdt and Michiels 2005), (Vogeleer, Tremblay et al. 2015), (Goulter, Gentle et al. 2010). Especially, cell surface hydrophobicity is generally associated with bacterial adhesiveness and biofilm formation (Mirani, Fatima et al. 2018), therefore the cell hydrophobicity values were measured using the BATH method. The

expression of *mcr-1* in ATCC 43894 decreased cell surface hydrophobicity. These results were consistent with the reduction of the biofilm formation of ATCC 43894.

In addition, *mcr-1* decreased swimming motility ability of ATCC 43894. It did not appear to be a function of growth, since there were no differences in growth rates of all strains. Generally, acquisition of antibiotic resistance entails a loss of bacterial fitness. Several studies reported that *E. coli* strains with *mcr-1*-harboring plasmids did not suffer a fitness cost (Tietgen, Semmler et al. 2018). which might be consistent with rapid dissemination of *mcr-1*-positive *E. coli* strains (Sun, Zhang et al. 2018), (Kusumoto, Ogura et al. 2016), (Elnahriry, Khalifa et al. 2016).

The results of bacterial adhesion and invasion assay showed that *mcr-1* increased bacterial adherence to HEp-2 cells while it decreased invasiveness of ATCC 43894. EHEC pathogenesis is not restricted to toxin-mediated effects, and a combination of virulence traits seems to be required, as demonstrated by the intimate bacterial attachment to host epithelial cells leading to the characteristic

attaching and effacing (A/E) lesions (Cordonnier, Etienne-Mesmin et al. 2017). The increase of adhesion of ATCC 43894 to human epithelial cells mediated by *mcr-1* may pose a lot of clinical problems.

Lipid A modification resulting from *mcr-1* by the addition of phosphoethanolamine in Gram-negative ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens were reported (Liu, Chandler et al. 2017). However, the results in this study showed that *mcr-1* modified the polysaccharide of LPS as well as lipid A. O-antigen of Gram-negative bacteria play a role in modulate the interaction with external factors, such as the components of the immune system and bacteriophage (Kulikov, Golomidova et al. 2019). Because bacteriophage use LPS or outer-membrane protein as a receptor to infect host bacteria, these modifications of LPS including polysaccharide may affect the bacteriophage infection. Also, these modifications of LPS may affect the outer-membrane. A recent study reported that impact of *mcr-1* expression on the membrane permeability of *E.* coli, since *mcr-1* modifies the structure of LPS (Li, Yin et al. 2020). Study of outer-

membrane alteration by *mcr-1* expression in *E. coli* are not well established, so further studies are required.

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국문초록

콜리스틴은 다제내성 그람 음성균에 사용되는 최후의 항생제로 여겨진다. 그러나 최근 중국에서 대장균에서 플라스미드 매개 콜리스틴 저항성 유전자인 mobilized colistin resistance(*mcr)-1*이 처음 보고되었다. 그 이후, 장출혈성 대장균과 같은 병원성 대장균에서의 플라스미드에 의한 *mcr-1* 유전자의 전파는 전세계적으로 많은 공중 보건 문제를 야기하고 있다. 한국에서도 식품, 사람, 그리고 가축에서 *mcr-1* 보유 플라스미드가 보고되었다. 전장 유전체 분석(whole-genome sequencing) 분석을 통해 *mcr-1* 보유 plasmid가 선모와 type VI secretion system과 같은 병원성 유전자들을 암호화하고 있는 것을 확인하였다. 이에 본 연구에서는 *mcr-1* 보유 플라스미드의 전달이 장출혈성 대장균의 병원성에 어떤 영향을 미치는지 알아보고자 하였다. *mcr-1* 보유 플라스미드는 접합에 의해 EHEC ATCC 43894로 쉽게 전달되었으며, 10일의 연속적인 계대배양에도 ATCC 43894에서 안정적으로 유지되었다. 한국을 포함한 아시아에서 많이 발견되는 IncI2 유형의 *mcr-1* 보유 플라스미드 20개에 대한 범-게놈 분석(Pan-genome analysis)을 기반으로, *mcr-1*을 포함하여 병원성과 관련이 있다고 알려진 유전자(*pilS* 및 *virB2*)를 선정하였으

며, 병원성 평가 연구를 수행하기 위해 선정된 유전자들의 돌연변이를 구축하였다. 그 결과, *pilS* 및 *virB2*는 ATCC 43894의 병원성에 영향을 끼치지 않았다. 흥미롭게도, *mcr-1*은 ATCC 43894의 생장에 영향을 미치지 않음에도 불구하고, ATCC 43894의 swimming motility를 감소시켰다. 또한, *mcr-1*은 ATCC 43894의 세포 표면의 소수성을 감소시켰으며, 이는 biofilm 형성을 저해하는데 영향을 끼쳤다. *mcr-1*은 사람 상피 세포인 HEp-2 세포에 대한 ATCC 43894의 adhesion을 증가시키며 invasion을 감소시켰다. Deoxycholate-polyacrylamide gel electrophoresis(DOC-PAGE)를 통해 *mcr-1*에 의한 core oligosaccharide-lipid A와 O antigen 영역의 밴드 패턴의 변화를 확인하였으며, 이러한 결과는 *mcr-1*이 기존에 보고된 lipid A 뿐 아니라 다당류의 구조 또한 변형시켰음을 나타낸다. 종합하면, 본 연구 결과는 ATCC 43894의 *mcr-1*의 획득이 콜리스틴 내성의 증가뿐만 아니라 병원성에도 영향을 미칠 수 있음을 보여준다.

주요어: *mcr-1*, 장출혈성 대장균, 항생제 내성

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