Study the Expression of *ompF* Gene in *Esherichia coli* Mutants

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The outer membrane porin proteins are the major factors in controlling the permeability of cell membrane. *OmpF* is an example of porin proteins in *Esherichia coli*. In normal growth condition a large amount of this protein is synthesised, but under stress condition, such as the presence of antibiotics in environment its expression is decreased inhibiting the entrance of antibiotics into cell. The expression of *ompF* is inhibited by antisense RNA transcribed from *micF*. In normal condition the expression of *micF* is low, but in the presence of antibiotics its expression is increased and causes multiple resistances to irrelevant antibiotics. The aims of this research were to study first, the intactness of *micF* and then quantify the expression of *ompF* in ciprofloxacin and tetracycline resistant mutants of *E. coli*. For this purpose the 5' end of *micF* was amplified and then sequenced. None of these mutants except one and its clone has a mutation in this gene. Then the relative expression of *ompF* in these mutants was quantified by real time PCR. There was no significant difference between *ompF* transcription of mutants and wild type strain. Based on this study and previous study it is concluded that low to intermediate levels of resistance to ciprofloxacin and tetracycline does not decrease *ompF* transcription.

Key words: *micF*, multiple antibiotic resistance, *ompF*, real time PCR

Small noncoding RNAs (snRNAs) are present in all organisms. They are antisense RNA that regulate post-transcriptionally gene expression and promote adaptation of cells to various growth conditions[1]. There are more than 80 snRNAs in *Esherichia coli* (*E. coli*) genome[2]. Among these snRNAs, some regulate the expression of outer membrane porins, such as *micC* and *micF* RNAs. *micF* RNA regulates negatively the expression of *ompF* in the presence of antibiotics such as tetracycline[3]. About 25 nucleotide (nt) at the 5′ end of *micF* RNA base pairs to *ompF* 5′ untranslated region (5′ UTR) mRNA and forms a duplex (fig. 1). As the formation of duplex is not perfect, the small stem loop structure produces. *micF* RNA in the duplex structure, covers the Shine-Dalgarno sequence (ribosome binding site) and AUG start codon of *ompF* mRNA and thereby inhibits its translation[3].

In addition to general transcriptional regulator, including H-NS, HU and Lrp, specific transcriptional regulator, such as MarA positively regulates *micF* transcription[4]. There is a mar box next to and partly overlapping the -35 region of the *micF* promoter[5]. *marA* is located in *marRAB* operon whose up and down regulations are under the control of MarA and MarR, respectively[5]. Binding of different ligands, such as antibiotics to MarR, dissociates this repressor from the operator site of *marRAB* operon[6]. Then binding of MarA to mar box upstream of -35 region of *marRAB* operon activates this operon expression[5,7]. Over activity of MarA enhances the transcription of *micF* and thereby decreases the translation of *ompF* mRNA[4]. To decrease the translation of *ompF* mRNA, an intact *micF* locus is required[8]. Additionally, expression of *ompF* is regulated at transcription level as well.

Outer membrane proteins (porins) such as *ompF* and OmpC are abundant proteins and form trimeric β-barrels in the OM[4]. They form channels through the outer membrane (OM) for the entry of different compounds for example nutrients and antibiotics, such as ciprofloxacin and tetracycline. Thus, decreased levels of *ompF* prevent the entry of above antibiotics to *E. coli* cells and cause multiple antibiotic resistance phenotype[4].

In the previous work *gyrA* mutants, which are resistant to ciprofloxacin and tetracycline, and with and without a mutation in *marR* were described[9,10]. These mutants and their increased tetracycline

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resistant clones could possess low level of *ompF*. The aims of this research were first, to study the intactness of *micF* gene and its mar box; and then to study *ompF* expression in these mutants.

MATERIALS AND METHODS

Tetracycline hydrochloride (Tc) (Sigma) was used to promote resistance in mutants. Stock solution of 4 mg/ml was prepared for the study. Diluted LB broth (Merck, Germany) and LBA containing 1.5% agar (Merck, Germany) were used for cultivation of strain and mutants.

Bacterial strain and mutants:

As defined previously resistance to ciprofloxacin can be divided to three levels, including low levels of resistance (MIC: 0.063 to 1 μg/ml), intermediate levels of resistance (MIC: 1 to 32 μg/ml) and high levels of resistance (MIC: >32 μg/ml)[11]. Additionally, it was described that resistance to tetracycline can also be divided into three levels, including low levels of resistance (MIC: 1 to 10 μg/ml), intermediate levels of resistance (MIC: 10 to 50 μg/ml) and high levels of resistance (MIC: >50 μg/ml)[12]. MG1655 was wild type and control strain. *gyrA* mutants with and without a mutation in *marR* gene, and based on above definition were with low to intermediate levels of resistance to ciprofloxacin and tetracycline isolated in previous work[10] are listed in Table 1. Mutants W25, W26 and W49 were isolated from cultivation of wild type strain on LBA plus ciprofloxacin[9]. Clones C6, C14 and C17 were obtained from cultivation of above mutants on LBA agar containing Tc (unpublished work).

PCR amplification and DNA sequencing of *micF* gene:

Colony PCR was used to amplify the 5′ end of *micF* gene and upstream sequences harboring mar box in wild type and mutants[10]. Primers for amplification are listed in Table 2. PCR products (148 bp) were sequenced and compared with MG1655 *micF* sequence obtained from NCBI.

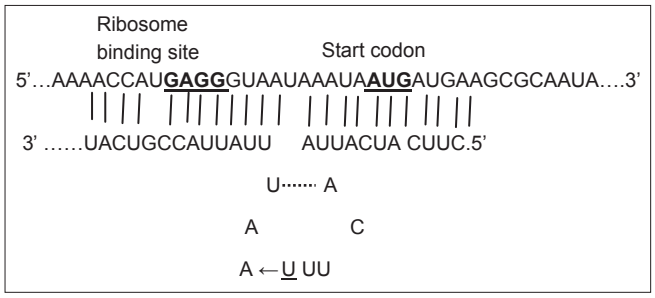

Fig. 1: Duplex formation between *micF* RNA and 5′UTR of *ompF* mRNA.
The underlined U is the nucleotide changed in one of mutants. Modified and adapted from Vogel and Papenfort, 2006.

OmpF expression analysis by real time PCR:

After cultivation of bacteria in diluted LB broth and 3 μg/ml Tc (except for wild type) at 37° with shaking at 150 rpm and grown to mid-logarithmic phase (OD_{600} of 0.6)[11]. Each culture was stabilised by RNA protect bacterial reagent (Qiagen, Germany) and then pelleted by centrifugation (Sigma, Germany). RNA was extracted immediately using an RNeasy Mini Kit (Qiagen, Germany), contaminating genomic DNA was eliminated by RNase-free DNase I treatment according to the manufacturer's instruction (Fermentas, Life science research, Vilnius, Lithuania) and its absence was confirmed by amplification of RNA samples plus a DNA sample as a positive control. Total RNA concentration was estimated at OD_{260} using spectrophotometer (Ultrospec 1100, Amersham Pharmacia Biothech, UK). Purified total RNA (2 μg) was used as a template in RT-PCR using a RevertAid Reverse Transcriptase kit (Fermentas, Life science research, Vilnius, Lithuania). The cDNAs obtained from reverse transcription were used to quantify the level of *ompF* and *gapA*, as an endogenous

TABLE 1: BACTERIAL STRAIN AND MUTANTS

Strain/ Mutant/ Clone	Relevant properties	MIC		Source/ Reference
		Cip (ng/ml)	Tc (μg/ml)	
MG1655	Wild type	35	3	A gift from Prof. Lloyd
W25	Wild type; *gyrA* and *marR*	75	4	[10]
W26	Wild type; *gyrA*	75	4	[10]
W49	Wild type; *gyrA* and *marR*	625	4	[10]
C6	W25; selected on tetracycline (5 μg/ml)	1000	45	Unpublished work
C14	W26; selected on tetracycline (5 μg/ml)	1000	30	Unpublished work
C17	W49; selected on tetracycline (5 μg/ml)	1000	30	Unpublished work

TABLE 2: LIST OF PRIMERS

Gene	Primer sequence (5′-3′)	Length of amplicon (bp)	Reference
micF	GGTTAAAATCAATAAC	148	This work
	GAAATAGGGGTAAAC		
ompF	CGTACTTCAGACCAGTAGCC	209	[14]
	GAACTTCGCTGTTCAGTACC		
gapA	ACTTACGAGCAGATCAAAGC	170	[14]
	AGTTTCACGAAGTTGTCGTT		

reference gene by real time PCR in a Rotor Gene 6000 thermocycler (Corbett Research, Australia) using a SYBR Green kit (Takara, Japan). Primers used in this experiment are listed in Table 2. Relative gene expression was calculated using the efficiency corrected calculation method (ratio of *ompF* expression to *gapA* expression, which was calculated by following equation, ratio=$(E_{ompF})^{\Delta Ct}/(E_{gapA})^{\Delta Ct}$…(1)[13]. The efficiency of each gene (E) can be obtained from linear regression plot which is drawn from serial dilution of standard sample and tested samples. The slope of the regression line was used in calculation of PCR efficiency, using equation $E=10^{[-1/slope]}$…(2). The difference in cycle threshold (Ct) was calculated using equation, $\Delta Ct=Ct_{wt}-Ct_{mutant}$…(3).

All data on *ompF* expression are the average of triplicate analyses. The data was recorded as mean±SD. Statistical analysis of relative expression was done by SPSS version 16 and *T*-test was used for comparison of relative gene expression data. A *P*-value of less than 0.05 is considered significant.

RESULTS

Mutations listed in Table 1 with different MIC for ciprofloxacin and tetracycline were analysed for the presence of possible mutation in mar box and 5′ end of *micF* gene involved in duplex formation with *ompF* mRNA. Fig. 2 shows the result of gel electrophoresis of the *micF* PCR products of MG1655 and mutants. The comparison of nucleotide sequence of PCR products with published sequence of *micF* showed that W26 and its derived clone, C14 had a nucleotide change ($T_{18}\rightarrow A$) in *micF* gene (fig. 3). This change is located in a region which participates in duplex formation with *ompF* mRNA (fig. 1). As can be seen from fig. 1, this alteration is located in stem loop structure of *micF* RNA following its base pairing with *ompF* mRNA. Other mutants showed the same sequence as MG1655. Moreover, the mar box of all mutants was intact (fig. 3).

As mutants used in this study were with or without *marR* mutation, it was possible that they reduce *ompF* expression. Purified RNAs were used for real time analysis. Results reveal that the slope of regression line was −3.1 and −3.5 for *gapA* and *ompF*, respectively. Thus, the efficiency of *ompF* and *gapA* were 1.94 and 2.1, respectively. The melting curve of two genes showed just one major peak which indicates the purity of samples. Fig. 4 shows the melting curve of *ompF* in wild type and mutants. The melting point of *ompF* and *gapA* were 87 and 86°C. Fig. 5 shows the amplification curve of *ompF* in wild type and mutants. Ct values ranged from 14 to 20. Table 3 shows the relative

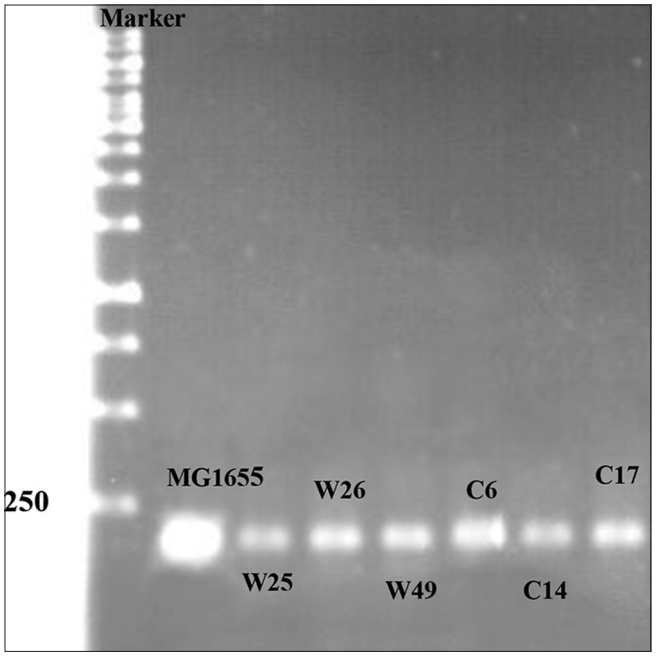

Fig. 2: Gel electrophoresis of PCR product.
First lane contains 1Kb DNA ladder and other lanes contain PCR products of wild type and mutants.

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MG1655   ACAGCACTGAATGTCAAAACAAAACCTTCACTCGCAACTAGAATAACTCCCGCTATCATC 60
W25      ACAGCACTGAATGTCAAAACAAAACCTTCACTCGCAACTAGAATAACTCCCGCTATCATC 60
W49      ACAGCACTGAATGTCAAAACAAAACCTTCACTCGCAACTAGAATAACTCCCGCTATCATC 60
C6       ACAGCACTGAATGTCAAAACAAAACCTTCACTCGCAACTAGAATAACTCCCGCTATCATC 60
C17      ACAGCACTGAATGTCAAAACAAAACCTTCACTCGCAACTAGAATAACTCCCGCTATCATC 60
W26      ACAGCACTGAATGTCAAAACAAAACCTTCACTCGCAACTAGAATAACTCCCGCTATCATC 60
C14      ACAGCACTGAATGTCAAAACAAAACCTTCACTCGCAACTAGAATAACTCCCGCTATCATC 60
         ************************************************************

MG1655          ATTAACTTTATTTATTACCGTCATTCATTTCTGAATGT 98
W25             ATTAACTTTATTTATTACCGTCATTCATTTCTGAATGT 98
W49             ATTAACTTTATTTATTACCGTCATTCATTTCTGAATGT 98
C6              ATTAACTTTATTTATTACCGTCATTCATTTCTGAATGT 98
C17             ATTAACTTTATTTATTACCGTCATTCATTTCTGAATGT 98
W26             ATTAACTTAATTTATTACCGTCATTCATTTCTGAATGT 98
C14             ATTAACTTAATTTATTACCGTCATTCATTTCTGAATGT 98
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Fig. 3: Multiple sequence alignment of 5′ end of *micF* gene and its upstream region in MG1655 (wild type) and mutants.
The underlined nucleotide sequences shown in order from left side are the mar box, RNP -10 signal and 5′ end of *micF* gene, respectively.

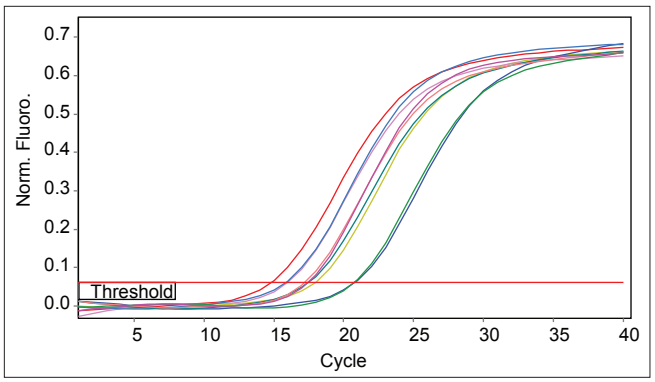

Fig. 4: Melting curves of *ompF* in wild type and mutants.
The yellow color curve belongs to wild type and other colored curves belong to mutants.

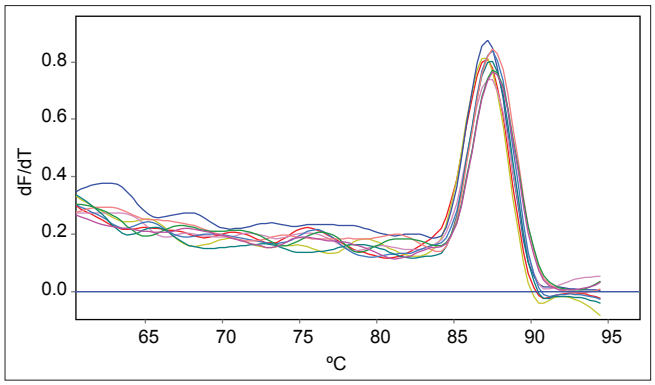

Fig. 5: Amplification curves of *ompF* in wild type and mutants. The pale blue curve belongs to wild type and other colored curves belong to mutants.

TABLE 3: RELATIVE EXPRESSION OF *OMPF* IN WILD TYPE (MG1655) AND MUTANTS

Strain/mutant/clone	Relative expression
Wild type (MG1655)	1±0
W25	0.8±0.01
W26	0.85±0.012
W49	0.756±0.02
C6	0.7±0.015
C14	0.71±0.02
C17	0.75±0.01

expression of *ompF* in these mutants. The *t*-test analysis showed no significant difference between wild type and mutants for expression of *ompF* ($P<0.05$). The reason for this result may be due to low to intermediate level of resistance to ciprofloxacine and Tc (Table 1).

DISCUSION

OmpF is an outer membrane porin found in gram negative bacteria, such as *E. coli*[3]. This porin is used for entrance of drugs, including quinolones, tetracycline and β-lactams[11]. It was said that down regulation of *ompF* causes resistance to multiple antibiotics, for example quinolones and tetracycline[11]. Low expression of *ompF* was frequently found in clinical isolates with high to intermediate levels of resistance to ciprofloxacine. These isolates contain alterations in genes encoding topoisomerase II (gyrase) and topoisomerase IV subunits, including *gyrA* and *parC*[11]. The expression of *ompF* is regulated at both transcriptional and translational levels. At translational level the expression of *ompF* is negatively controlled by *micF* RNA, a small antisense RNA that base pairs with *ompF* RNA[3]. The expression of *micF* is increased by transcriptional activator called MarA in the presence of ciprofloxacine or tetracycline. Thus, it is expected that the synthesis of *OmpF* is decreased[14]. However, it was demonstrated that the down regulation of *OmpF* is not completely dependent on up regulation of MarA activity[15].

In addition, it was shown that salicylate also reduces the translation of *ompF* RNA mainly by the mechanism similar to antibiotics[16]. On the other hand, at transcriptional level the two component regulatory system, EnvZ-OmpR located in cell membrane is responsible. However, this regulation is dependent on external osmolarity not the presence of antibiotics; in the way that low osmolarity causes *ompF* up regulation[17]. To see whether the transcription of *ompF* is changed in the presence of tetracycline, the level of *ompF* mRNA was quantified by real time PCR in mutants with low to intermediate levels of resistance to ciprofloxacin and tetracycline. Also the intactness of *micF* was checked in these mutants by PCR amplification of 5′ end and upstream region of this gene and sequencing the PCR products to ensure that they maintain the ability to base pair with *ompF* RNA following the up regulation of MarA in the presence of antibiotics.

Moreover, as mentioned before for *marA* dependent reduction of *OmpF* synthesis, an intact *micF* locus is required[8]. This includes and intact mar box and 5′ end of *micF* gene[3,5]. It was found that in all mutants except one (W26) and its clone (C14) the sequence of 5′ end of *micF* is intact. In W26 and its derived clone (C14) the nucleotide change in *micF* sequence would be in small loop structure following its attachment to *ompF* RNA. Possibly, it may not affect on base pairing ability. However, further study is needed to prove it.

Furthermore, statistical analysis did not reveal any significant difference between *ompF* expression in mutants and wild type strain ($P<0.05$). This is consistent with previous results on multidrug resistant mutants isolated from calves[18]. Reduced expression of *ompF* was seen in *E. coli* mutants with high levels of resistant to ciprofloxacin[11]. However, reduction in *ompF* expression was not seen in all mutants with low to intermediate levels of resistance to ciprofloxacin[11]. Kishii *et al.*[11] suggest that different genetic backgrounds are the cause of low or normal expression of *ompF* in mutants with low and intermediate levels of resistant to ciprofloxacin. Also, it is possible that a change in unknown factor is also necessary for down regulation of *ompF*[15].

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