Molecular analysis of porin gene transcription in heterogenotypic multidrug-resistant *Escherichia coli* isolates from scouring calves

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Background: Despite evidence that altered membrane porins may impair microbial drug uptake thereby potentially compounding efflux pump-mediated multidrug resistance, few studies have evaluated gene transcription to identify multidrug-resistance-associated porins and other potential drug targets.

Methods: Genes that encode six membrane porins (*fadL, lamB, ompC, ompF, ompW* and *yiaT*) and two membrane proteins (*tolC* and *ompT*) were assessed by PCR and by quantitative real-time PCR (qRT–PCR) analysis of 10 multidrug-resistant (MDR) and 10 antibiotic-susceptible (AS) *Escherichia coli* isolates. The mean ΔCt values for the study *E. coli* genes were analysed by the Wilcoxon test (P=0.05).

Results: All 20 *E. coli* isolates tested positive for *tolC, lamB, ompC, ompF* genes, while 10 MDR and 9/10 (90%) AS isolates were positive for the *fadL* gene. Seven out of 10 (70%) MDR and 7/10 (70%) AS isolates were positive for the *yiaT* gene, while 7/10 (70%) MDR and only 4/10 (40%) AS isolates were positive for the *ompT* gene. The mean ΔCt values for the *tolC* and *yiaT* genes were significantly higher in MDR than in AS isolates (Wilcoxon test; P<0.05). No significant difference was seen with respect to *fadL, lamB, ompC, ompF, ompT* and *ompW* gene transcription (Wilcoxon test; P>0.05).

Conclusions: Findings suggest up-regulated transcription of *tolC* and *yiaT* genes in the MDR *E. coli* isolates. These results indirectly suggest that TolC and YiaT proteins may play some role(s) in multidrug resistance, but proteomic studies are needed before the two proteins are considered potential drug targets.

Keywords: qRT–PCR, TolC, YiaT

Introduction

Multidrug resistance is a serious public and animal health problem that presents a major challenge to the treatment of bacterial infections. Recently, various species of extensively drug-resistant (XDR) bacteria including important pathogens like *Mycobacterium* spp., various members of the family *Enterobacteriaceae*1–6 and other XDR bacterial strains such as *Pseudomonas aeruginosa*7 and *Acinetobacter baumannii*8 have been reported. These reports underscore the challenge posed by these pathogens to antimicrobial therapy of human and animal disease. Since drug molecules must cross the bacterial cell membrane and wall before reaching the target site, the outer membrane of Gram-negative bacteria presents a considerable physical barrier to the influx of drug molecules. Other general mechanisms of bacterial resistance include, but are not limited to, reduced transmembrane diffusion of drug molecules, mutation of the drug target and enzymatic modification or degradation of the antibacterial agent.9 It is widely known that increased expression of membrane-based efflux pumps (EPs) is associated with multidrug resistance in most clinically relevant bacterial pathogens.10–13 This is because EPs expel a wide range of structurally unrelated compounds including various antibacterial agents.10–13 The outer membrane of Gram-negative bacteria also contains protein channels called porins that serve as conduits through which various compounds including several groups of antibacterial agents gain access to the interior of the organism.19–21 Interaction of antibiotic molecules and porin channels determines drug translocation efficiency; and likewise, the ability of bacteria to reduce the influx of antibiotic molecules through altered membrane porins contributes to the emergence of antibiotic resistance.20–22
A number of researchers have reported that impaired uptake of the drug molecules due to altered porins may compound EP-mediated drug resistance.\textsuperscript{20,21,23,24} In fact, various researchers have reported altered porin expression in drug-resistant Gram-negative bacteria.\textsuperscript{4,18,25,26} Clinically significant multidrug resistance related to altered membrane permeability has been reported in Gram-negative enteric bacteria.\textsuperscript{20,21} These researchers specifically document porin loss as a major bacterial resistance mechanism that restricts the influx of β-lactam and fluoroquinolone antibiotics.\textsuperscript{20,21} Porin modification, which may involve either up- or down-regulation of porin genes, has also been cited as a mechanistic event in the development of drug resistance.\textsuperscript{5,8,25,27}

ToIC is a ubiquitous outer membrane protein (OMP) that participates in the assembly of tri-component EP systems in Gram-negative bacteria.\textsuperscript{10,14–16,28,29} That allows various substrates to be transported from the inside to the outside of the microbial cell. Along with ToIC, \textit{Escherichia coli} expresses a number of trimeric porins otherwise called ‘the classical porins’. These include outer membrane protein F (OmpF) and outer membrane protein C (OmpC).\textsuperscript{23,30,31} Down-regulation of porin expression has been associated with drug resistance in \textit{Pseudomonas cepacia},\textsuperscript{32} while an ompF-null mutant \textit{Salmonella enterica} serovar Typhi isolate was also found to be resistant to chloramphenicol.\textsuperscript{33} Likewise, several strains of drug-resistant \textit{Haemophilus influenzae} with decreased porin gene expression have been reported.\textsuperscript{34} Whereas drug-resistance-associated down-regulated porins have been widely documented, a Chinese research team recently reported up-regulation of ToIC and the membrane porins OmpC and OmpW, along with simultaneous down-regulation of the porins OmpF and FadL in a nalidixic acid-resistant \textit{E. coli} isolate.\textsuperscript{27} The same workers also reported up-regulation of ToIC and the porin LamB, and down-regulation of FadL and OmpW in streptomycin-resistant \textit{E. coli} isolates.\textsuperscript{25} These observations have justified mechanistic studies into the dynamics of porin gene regulation and how altered expression of porin and other OMP genes relates to drug resistance, with the hope that the new knowledge can be used in the design of novel efficacious treatments for infections caused by multidrug-resistant (MDR) pathogens. Our research group speculates that in the long term, certain membrane porins may serve as potential targets for novel drugs with efficacy against MDR/XDR bacterial pathogens. However, there is need for a thorough understanding of porin gene transcription and expression dynamics in MDR/XDR bacteria so that the porin and other membrane proteins with the greatest relevance to drug resistance are targeted for drug development. Recently, a porin-targeting vaccine designed by scientists at Epitopix\textsuperscript{6} in MN, USA was licensed to prevent colonization of cattle with \textit{E. coli} O157:H7 (http://www.epitopix.com). Despite numerous studies documenting altered porin gene expression in drug-resistant bacteria,\textsuperscript{9,26,27} comprehensive studies have not been performed to evaluate porin gene expression in MDR bacteria. Therefore, the present research was designed to assess the relative transcription rates of genes that encode six membrane porins (FadL, LamB, OmpC, OmpF, OmpW and YiaT), a component of trimeric drug efflux pumps (ToIC) and a drug resistance-related membrane protease (OmpT) in MDR \textit{E. coli} isolates. Plans are underway to undertake translational proteomic studies of the corresponding proteins.

### Materials and methods

#### Study \textit{E. coli} isolates

All 10 MDR and 9 antimicrobial-susceptible (AS) \textit{E. coli} strains used in this research were isolated from faecal samples and/or intestinal contents from scouring calves at the North Dakota State University (NDSU) Veterinary Diagnostic Laboratory (VDL). The scouring neonatal calves from which the study isolates were cultured were raised at various beef and dairy farms located within the State of North Dakota as well as western parts of Minnesota. The AS1 isolate (ATCC \textit{E. coli} strain #25922) used as the experimental control was an ATCC strain (Manassas, VA, USA) with known broad-spectrum antimicrobial agent susceptibility. \textit{E. coli} isolates that were resistant to >80% of the antimicrobial agents tested were designated MDR and then selected for the study. The Sensititre panel included the following antibacterial agents: ampicillin; cefetamet; cefotaxime; chloramphenicol; clindamycin; danofloxacin; florfenicol; gentamicin; neomycin; oxytetracycline; penicillin; spectinomycin; sulfadimethoxine; tiamulin; tilmicosin; trimethoprim/sulfamethoxazole; tulasnychumycin; and tylosin–tartrate base. All the \textit{E. coli} isolates studied are currently kept in cryopreservation at −80°C in 20% glycerol and Luria–Bertani (LB) broth at Research Laboratory 154, Department of Veterinary and Microbiological Sciences, NDSU. The antimicrobial susceptibility profiles of the study \textit{E. coli} isolates were determined by the Bovine/Porcine Tulasnychumycin MIC Sensititre microplate using the Sensititre susceptibility system (Trek Diagnostic Systems, OH, USA) according to the manufacturer’s instructions.

#### Extraction of bacterial DNA

\textit{E. coli} isolates were inoculated on MacConkey agar plates and incubated overnight at 37°C. A single colony of each isolate was added to 40 μL of 10 mM Tris/1 mM EDTA (TE) buffer with 1% 20 mg/mL proteinase K. Samples were incubated at 55°C for 10 min followed by 10 min incubation at 80°C. The extracted DNA was then diluted with 80 μL of sterile water, centrifuged for 5 min and stored at −20°C for future PCR analysis.

#### PFGE

The genetic/clonal relationship between each of the study isolates in the two \textit{E. coli} groups was determined by PFGE analysis. The molecular size standard \textit{Salmonella Braenderup} BAA-664 (ATCC) along with \textit{E. coli} test cultures were streaked out on LB agar (Sigma, St Louis, MO, USA) and incubated overnight at 37°C. A sterile swab was used to transfer bacteria to a tube containing 2 mL of cell suspension buffer (100 mM Tris/100 mM EDTA, pH 8.0) and the concentration was adjusted to an absorbance reading of ≏1.35 at a wavelength of 610 nm. Seakem Gold agarose (Lonza, Rockland, ME, USA) was prepared as a 1% solution in TE buffer (pH 8.0) and stored in a 58°C water bath. A 400 μL aliquot of the bacterial suspension was gently mixed with 20 μL of proteinase K (20 mg/mL stock solution) in a 1.5 mL microcentrifuge tube. An equal volume of the preheated agarose was then added to the bacterial suspension, gently mixed and immediately dispensed into plug moulds (Bio-Rad Laboratories, Hercules, CA, USA). After the plugs solidified, they were placed into a 50 mL tube containing 5 mL of cell lysis buffer (50 mM Tris/50 mM EDTA/1% Sarkosyl, pH 8.0) along with 25 μL of proteinase K (20 mg/mL stock). Samples were allowed to incubate at 54°C for 2 h at 180 rpm in a shaking incubator. Following incubation, the cell lysis buffer was poured off and 10 mL of sterile water preheated to 50°C was added. The samples continued to incubate at 50°C in a shaking incubator for 15 min at 180 rpm. The water was then poured off and the water rinse was repeated followed by four washes with TE buffer (pH 8.0). An ≏2.0 mm wide slice of plug was cut with a single-edge razor blade and transferred to a 1.5 mL microcentrifuge tube containing...
100 μL of 30 U of XbaI (Promega Corporation, Madison, WI, USA) in 1x restriction buffer and 1.0 μL of BSA (Promega Corporation), followed by incubation at 37°C for 2 h. After incubation, the restriction digestion mixture was replaced with 200 μL of 0.5x Tris/borate/EDTA (TBE) and allowed to incubate at room temperature for 5 min. Electrophoresis was then carried out on the CHEF-mapper system (Bio-Rad Laboratories) using a 1% SeaKem Gold agarose gel and 0.5× Tris running buffer using the following electrophoresis conditions: initial switch time, 2.16 s; final switch time, 83.8 s; run time, 18 h; 14°C; and ramping factor linear. Upon completion of electrophoresis, the gel was stained for 30 min in an ethidium bromide solution (1 mg/mL) and destained with double distilled water. Samples were further characterized using the BioNumerics software (Applied Maths, Austin, TX, USA).

**PCR assay**

The PCR assay for the porin genes was carried out according to the conditions shown in Table 1. Primers used in this study (Table 2) were designed based on specific porin and OMP gene sequences obtained from the EcoCyc Database for *E. coli* K-12, strain MG1655 (SRI International) and manufactured by Trilink Biotechnologies. The primers include a housekeeping gene, *gapA*, used for a quantitative control. Working concentrations were determined by testing each primer at 300, 600 and 900 nM for each of the target genes. Each PCR reaction contained 5 μL of cDNA, 12.5 μL 2x Quantitect SYBR Green PCR master mix, the appropriate concentration of primer (Table 2) and 72°C for 30 s. All test samples were run in duplicate and four reactions without reverse transcriptase (RT negative) were prepared for each of the RNA samples. The cDNA synthesis was completed on a DNA Engine Thermocycler (Bio-Rad Laboratories) after which the resulting cDNA product was diluted 10-fold, and a 1:100 dilution was used in the qRT–PCR.

The Quantitect SYBR Green PCR Kit (Qiagen) was used for all real-time PCRs in accordance with the manufacturer’s instructions. Primers used in this study (Table 2) were designed based on specific porin and OMP gene sequences obtained from the EcoCyc Database for *E. coli* K-12, strain MG1655 (SRI International) and manufactured by Trilink Biotechnologies. Each primer pair was tested by real-time PCR. The primers include a housekeeping gene, *gapA*, used for a quantitative control. Working concentrations were determined by testing each primer at 300, 600 and 900 nM for each of the target genes. Each PCR reaction contained 5 μL of cDNA, 12.5 μL 2x Quantitect SYBR Green PCR master mix, the appropriate concentration of primer (Table 2) and 72°C for 30 s. All test samples were run in duplicate and four reactions without reverse transcriptase (RT negative) were prepared for each of the RNA samples. The cDNA synthesis was completed on a DNA Engine Thermocycler (Bio-Rad Laboratories) after which the resulting cDNA product was diluted 10-fold, and a 1:100 dilution was used in the qRT–PCR.

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**Statistical analysis**

The statistical analysis on the ΔΔCt values obtained by qRT–PCR was performed using SAS Version 9.13. Descriptive statistics entailed numerical summaries and graphs. A non-parametric approach Wilcoxon rank sum test was used to compare the location parameters of the ΔΔCt values for the two groups of *E. coli* (MDR and AS *E. coli* isolates). All tests were done at a 5% level of significance.
Porin gene transcription in multidrug-resistant \textit{E. coli}

\textbf{Table 2.} Working concentrations of the primers used in the qRT–PCR assay for porin and membrane protease genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Reference</th>
<th>Primer sequence (5′→3′)</th>
<th>Concentration (nM)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gapA-F</td>
<td>18</td>
<td>AACTCAGCAAATATCGTGGC CGGGATGATGGTTCTGGAAA</td>
<td>300</td>
<td>53</td>
</tr>
<tr>
<td>gapA-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ToIC-F</td>
<td>this study</td>
<td>ACAAACGGCAAGCAAGACGC CCACTACATAGCAATAGC</td>
<td>600</td>
<td>60</td>
</tr>
<tr>
<td>ToIC-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LamB-F</td>
<td>this study</td>
<td>GGTTCACATGTCGCGATTCG GGTGCCGTGTGTGTTATCC</td>
<td>300</td>
<td>60</td>
</tr>
<tr>
<td>LamB-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FadL-F</td>
<td>this study</td>
<td>CTCTTATACCTCTAAACTATGG TACGTCAACCCGTTGG</td>
<td>900</td>
<td>53</td>
</tr>
<tr>
<td>FadL-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YiaT-F</td>
<td>this study</td>
<td>CCGGTCGCGAGTAGTTATCC GCTGTGGTAATCTCTTCC</td>
<td>600</td>
<td>54</td>
</tr>
<tr>
<td>YiaT-R</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>OmpC-F</td>
<td>this study</td>
<td>CAGAGATGGGTCTCTTGC GAGTCTAGGTTACGAGG</td>
<td>600</td>
<td>53</td>
</tr>
<tr>
<td>OmpC-R</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>OmpF-F</td>
<td>this study</td>
<td>ACCCTGGCAAGCAACTACG AACCTACCCGATACATCG</td>
<td>600</td>
<td>54</td>
</tr>
<tr>
<td>OmpF-R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OmpT-F</td>
<td>this study</td>
<td>TCTCAAGCAACCCCAATACC TCTCACAGACCAAGATAGG</td>
<td>600</td>
<td>54</td>
</tr>
<tr>
<td>OmpT-R</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>OmpW-F</td>
<td>this study</td>
<td>GGGGACCGAACACATGG TGTTGCGGATGATGACG</td>
<td>300</td>
<td>53</td>
</tr>
<tr>
<td>OmpW-R</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Note that \textit{ompT} is a gene that encodes the membrane-based protease OmpT.

**Results**

\textbf{PFGE}

PFGE (Figure 1) did not indicate a clonal relationship between any of the isolates tested. The data indicate that all the study \textit{E. coli} isolates were distinct from each other, thus none of the data is a duplicate of any other isolates.

\textbf{Routine PCR}

Analysis of PCR products showed the relevant bands for the OMP and porin genes \textit{tolC} (197 bp), \textit{fadL} (294 bp), \textit{yiaT} (136 bp), \textit{lamB} (111 bp), \textit{ompC} (162 bp) and \textit{ompF} (191 bp) and for the membrane protease gene \textit{ompT} (198 bp) in all the MDR and AS \textit{E. coli} isolates (Figure 2). With reference to results of routine PCR, all MDR and AS \textit{E. coli} isolates had detectable \textit{tolC}, \textit{lamB}, \textit{ompC} and \textit{ompF} genes (Figure 2). PCR products for all the studied outer-membrane-encoding genes were also detected in all of the 10 MDR strains, while the \textit{fadL} gene was detected in 9/10 AS \textit{E. coli} isolates (Figure 2). Seven out of 10 (70%) MDR and 7/10 (70%) AS \textit{E. coli} isolates tested positive for the \textit{yiaT} gene (Figure 2), while 7/10 MDR isolates and 4/10 AS \textit{E. coli} isolates were positive for the \textit{ompT} gene (Figure 2). All the PCR results were mirrored by the individual \(\Delta \Delta Ct\) values [see the Supplementary data, available at JAC Online (http://jac.oxfordjournals.org/)] for each of the porin genes as determined by qRT–PCR.

\textbf{qRT–PCR}

The mean \(\Delta \Delta Ct\) values for the study porin and the other two membrane protein genes in the 20 study \textit{E. coli} isolates (10 MDR and 10 AS \textit{E. coli} isolates) were computed, and the Wilcoxon test \((P=0.05)\) was used to determine the level of statistical significance between the gene transcription in the two \textit{E. coli} groups. Based on the qRT–PCR data, the mean \(\Delta \Delta Ct\) values for the \textit{tolC} \((P=0.0002436)\) and \textit{yiaT} \((P=0.0041)\) genes were significantly higher in the MDR \textit{E. coli} isolates than in the AS \textit{E. coli} isolates, but no significant difference was seen for the \textit{fadL} \((P=0.3154)\), \textit{lamB} \((P=0.1903)\), \textit{ompC} \((P=0.1716)\), \textit{ompF} \((P=0.5787)\), \textit{ompT} \((P=0.2303)\) and \textit{ompW} \((P=0.1903)\) genes (Table 3). Of the two membrane protein genes that were apparently significantly hypertranscribed in the MDR isolates, \textit{tolC} showed the highest level of significance \((P=0.0002436)\) followed by \textit{yiaT} \((P=0.0041)\). The mean \(\Delta \Delta Ct\) values for the other OMP-encoding genes appeared higher, but statistical analysis did not reveal any significant difference between the transcription of MDR and AS \textit{E. coli} isolates.

**Discussion**

PFGE data did not indicate a significant clonal relationship between the \textit{E. coli} isolates tested during the study. Of the six porin and two OMP genes studied, the mean \(\Delta \Delta Ct\) values for the \textit{tolC} and \textit{yiaT} genes were significantly higher \((P<0.05)\) in MDR than for the AS \textit{E. coli} isolates, while that of the \textit{ompT} gene was borderline (Table 3). Interestingly, no statistically significant difference \((P>0.05)\) was seen in the transcription levels of \textit{fadL}, \textit{lamB}, \textit{ompC}, \textit{ompF} and \textit{ompW} genes even though the sum of the scores was higher than what was expected under the null hypothesis of no difference. Overall, these findings are consistent with up-regulated transcription of
tolC and yiaT genes, but not the other six OMP genes in the MDR E. coli isolates. Except for the yiaT gene, altered porin-gene-associated drug resistance has been investigated for the other seven proteins in drug-resistant E. coli isolates, but not in MDR bacteria. The present findings strongly suggest, albeit indirectly, that expression of TolC and YiaT proteins may be crucial in mediating MDR mechanisms. Plans are underway to study both proteins by translational proteomics as a prelude to evaluating their suitability as potential targets for developing efficacious drugs against potentially deadly MDR pathogens. Based on routine PCR data, the tolC gene was present in all study MDR and AS E. coli isolates (Figure 2). However, when the qRT–PCR data were analysed, the mean ΔΔCt value for the gene was significantly higher in the MDR isolates (P<0.05) than in the AS E. coli isolates (Table 3 and Figure 3). TolC has been widely reported for its drug efflux properties and plays a critical role in mediating clinically significant MDR. Up-regulation of TolC and other membrane proteins was also recently reported in E. coli isolates with resistance to chloramphenicol, streptomycin and nalidixic acid. As would have been expected, therefore, the present data strongly suggest, albeit indirectly, that possible TolC overexpression may be an important mechanism of MDR in Gram-negative bacteria. These findings need to be corroborated by translational proteomics to explore the potential for developing TolC-targeting therapeutic agents with efficacy against MDR/XDR Gram-negative bacteria.

Routine PCR analysis of the genes that encode the study membrane proteins revealed that 2/10 MDR and 3/10 AS E. coli isolates were negative for the yiaT gene. On the other hand, the qRT–PCR data were remarkably consistent with up-regulated yiaT transcription in MDR E. coli isolates in contrast to extremely low transcription levels in the AS E. coli isolates. YiaT is a 27.4 kDa protein that was first reported as a putative outer membrane porin. Despite the current knowledge of its structural identity and spatial location within the bacterial cell, the physiological and other biochemical functions of the protein have not been described to date. For the first time, data from our research demonstrate that up-regulated transcription of the yiaT gene may be associated with yet to be described drug resistance mechanisms. Subject to corroboration by translational proteomics, the present findings further suggest, albeit indirectly, that this porin may play some role(s) in MDR mechanisms. Since YiaT protein is predicted to be related to transmembrane substrate transport, these mechanisms deserve to be unravelled and characterized before the protein is fully considered a potential target for developing new drugs against XDR/MDR pathogens. However, the fact that a minority of both MDR and AS E. coli isolates did not transcribe the yiaT gene suggests that if it were to play a role in drug resistance mechanisms in MDR E. coli, the mechanism in question would perhaps be an adjunct to other systems. This finding is not surprising since most membrane porins are physiologically redundant and work together in multi-component networks. For example, the expression of OmpC and OmpF is modulated by the transcription regulators OmpR and EnvZ, and both are encoded by the ompB operon. What needs to be defined for YiaT are the possible regulatory pathways involved in its expression in addition to determining the precise manner in which those expression patterns may affect outer membrane permeability and drug resistance.

FadL is an outer membrane β-barrel protein involved in the uptake of long-chain fatty acids. The amino terminus of the
Porin gene transcription in multidrug-resistant *E. coli*

 Protein also contains an attachment site for bacteriophage T2, while the carboxyl end has a crucial amino acid sequence needed for fatty acid transport. Based on digital analysis of the relevant two-dimensional gel protein spot in a single *E. coli* isolate, a Chinese research team recently reported down-regulation of *fadL* expression in nalidixic acid- and streptomycin-resistant *E. coli* isolates. The qRT–PCR data from the present study did not demonstrate a statistically significant difference in *fadL* gene transcription rates between the MDR and AS *E. coli* groups.

Routine PCR analysis showed *ompC* expression by all 20 study *E. coli* isolates, while qRT–PCR analysis did not reveal a statistically significant difference between the mean ΔΔCt values for the 10 MDR and 10 AS *E. coli* isolates. OmpC is a small trimeric porin through which transmembrane transport of small ions and other hydrophilic solutes ≤500 Da takes place. The up-regulation of OmpC has been reported in drug-resistant bacteria, while decreased production of the protein has also been observed in response to exogenous polyanines. The latter compounds are known to induce resistance to ColE7, a product of an SOS regulon that encodes a bacteriocin with potency against susceptible *E. coli* and related enterobacteria under conditions of stress. The rather conflicting findings

**Figure 2.** Analysis of porin and membrane protease gene PCR products in 1% agarose gels stained with ethidium bromide. Lanes 1–10 represent MDR *E. coli* isolates and lanes 11–20 represent AS *E. coli* isolates. Note that duplicate lanes were loaded for each of the samples.
by Li et al.\textsuperscript{25} and our data are not surprising since current evidence suggests that progression of drug resistance mechanisms like those caused by continued exposure to subinhibitory concentrations of certain antimicrobial agents may in a step-by-step manner select for porin expression modifications that eventually lead to decreased expression or total OmpC loss.\textsuperscript{18} Li et al.\textsuperscript{25} have suggested that this porin along with TolC may play important roles in drug resistance mechanisms. However, their conclusions on OmpC were based on digital analysis of relevant gel spots from a single drug-resistant \textit{E. coli} strain.\textsuperscript{25} Whereas

<table>
<thead>
<tr>
<th>Primer</th>
<th>No. of MDR \textit{E. coli} isolates</th>
<th>No. of AS \textit{E. coli} isolates</th>
<th>Wilcoxon statistic</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>tolC</td>
<td>10</td>
<td>10</td>
<td>148</td>
<td>0.0002436**</td>
</tr>
<tr>
<td>lamB</td>
<td>10</td>
<td>10</td>
<td>123</td>
<td>0.1903</td>
</tr>
<tr>
<td>ompC</td>
<td>10</td>
<td>10</td>
<td>123</td>
<td>0.1716</td>
</tr>
<tr>
<td>fadL</td>
<td>10</td>
<td>9</td>
<td>77</td>
<td>0.3154</td>
</tr>
<tr>
<td>yiaT</td>
<td>7</td>
<td>7</td>
<td>74</td>
<td>0.0041*</td>
</tr>
<tr>
<td>ompF</td>
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<td>10</td>
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</tr>
<tr>
<td>ompT</td>
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<td>4</td>
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<td>0.2303</td>
</tr>
<tr>
<td>ompW</td>
<td>10</td>
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<td>123</td>
<td>0.1903</td>
</tr>
</tbody>
</table>

A statistically significant difference was verified for the transcription levels of the \textit{tolC} and \textit{yiaT} genes (\( P < 0.05 \)), but not for \textit{fadL}, \textit{lamB}, \textit{ompC}, \textit{ompF}, \textit{ompT} and \textit{ompW} genes (\( P > 0.05 \)). A single asterisk indicates a statistically significant \( P \) value, while double asterisks denote a highly significant \( P \) value.

Figure 3. Bar graphs showing the mean \( \Delta\Delta Ct \) values for the \textit{yiaT}, \textit{ompT}, \textit{tolC}, \textit{lamB}, \textit{ompC}, \textit{fadL}, \textit{ompW} and \textit{ompF} genes in the 10 MDR and 10 AS \textit{E. coli} isolates.
current evidence on the unequivocal role of TolC in multidrug resistance justifies targeting it for the development of novel drugs, the conflicting results on OmpC transcription dynamics suggest that despite the potential role in drug resistance mechanisms, targeting this protein may be problematic and unrewarding. This is due to the consideration that both up-regulation and down-regulation may have mechanistic implications for drug resistance.

Routine PCR analysis showed the presence of the \textit{ompF} gene in all 20 study \textit{E. coli} isolates, while qRT–PCR analysis revealed no statistically significant difference between the mean \textit{DD\textsubscript{Ct}} values for the 10 MDR and 10 AS \textit{E. coli} isolates. OmpF is a large porin that allows transmembrane diffusion of solutes such as sugars, ions and amino acids \textless 600 Da in size.\textsuperscript{30,43} Loss of large-channel porins is often associated with increased resistance to hydrophilic antibacterial agents.\textsuperscript{33} For instance, \textit{ompF} \textit{E. coli} mutant strains have been reported to develop resistance to \(\beta\)-lactam antibiotics,\textsuperscript{34} while clinical isolates of porin-deficient \textit{Serratia marcescens} were resistant to aminoglycosides and \(\beta\)-lactam antibiotics.\textsuperscript{65} An \textit{ompF}–null mutant \textit{Salmonella enterica} serovar Typhi isolate was also resistant to chloramphenicol.\textsuperscript{33} Under physiological conditions, OmpF imports group A colicins, the bactericidal molecules produced by certain \textit{E. coli} isolates.\textsuperscript{29} Other studies have shown that OmpF production may decrease in response to exogenous polyamines.\textsuperscript{22} The latter have been shown to inhibit the influx of antibiotic molecules into bacteria.\textsuperscript{21} Most recently, down-regulation of \textit{ompF} expression was reported in a drug-resistant \textit{E. coli} isolate.\textsuperscript{25}

In the present study, routine PCR analysis showed the presence of the \textit{lamB} gene in all 20 \textit{E. coli} isolates. The mean \textit{DD\textsubscript{Ct}} values obtained by qRT–PCR analysis of the 10 MDR \textit{E. coli} were higher than for the 10 AS \textit{E. coli} isolates, but this difference was statistically insignificant. LamB is a sugar-specific porin involved in transmembrane transport of maltose and maltdextrins that are crucial for bacterial metabolism.\textsuperscript{46} This porin was reported to be down-regulated in a tetracycline-resistant \textit{E. coli} isolate,\textsuperscript{47} but was also later shown to be up-regulated in a streptomycin-resistant \textit{E. coli} isolate.\textsuperscript{25} Our finding with regard to the presence of the gene in all 20 \textit{E. coli} isolates studied is neither surprising nor of much significance.

Transcription dynamics of the \textit{ompT} gene in the two \textit{E. coli} groups were also evaluated and found to be borderline. This gene encodes an outer membrane protease that is known to hydrolyse the antimicrobial peptide protamine.\textsuperscript{48} Recently, up-regulation of OmpT was reported in a drug-resistant \textit{E. coli} isolate\textsuperscript{25,36} suggesting it may play a role in drug resistance most probably through degradation of antibiotic molecules.
The membrane protease has also been reported to be a virulence factor in urinary tract infections. Based on this background, we wanted to assess the transcription dynamics of the ompT gene in MDR E. coli isolates to determine whether membrane-based enzyme might indeed participate in drug resistance mechanisms. In the present study, routine ompT PCR results were the most dramatic as 7/10 of the MDR and only 4/10 AS E. coli strains had a detectable PCR signal for the ompT gene.

The ultimate goal of this research was to predict which membrane porins or protease/protein might be involved in MDR mechanisms so that new targets for developing efficacious anti-MDR pathogen drugs are identified. Experimental data indicated for the first time that the novel porin YiaT is likely to be associated with the MDR phenomenon. The findings of our research suggest, albeit indirectly, that in addition to TolC, the porin YiaT may play a role in mediating MDR mechanisms. As such, the two OMPs should be studied further by translational proteomics and explore their potential as targets for developing novel drugs against infections caused by troublesome MDR pathogens. Subject to corroboration by translational proteomics of the study OMPs, the current qRT–PCR data appear to suggest that transcriptional differences within the lamB, ompC, ompF and ompW genes may not be associated with MDR mechanisms.

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Transparency declarations
None to declare.

Supplementary data
Supplementary data are available at JAC Online (http://jac.oxfordjournals.org/).

References
Porin gene transcription in multidrug-resistant E. coli