Involvement of MarR and YedS in carbapenem resistance in a clinical isolate of *Escherichia coli* from China

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Running title: MarR and YedS in a carbapenem-resistant *E. coli* isolate

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Abstract: A carbapenem resistant clinical isolate of *Escherichia coli*, which lacked OmpF and OmpC porins, carried a *marR* mutation and expressed a functional YedS, a normally non-translated gene. MarR and YedS are described here as having effects on the ability of this strain to resist carbapenems. Additionally, expression of YedS was regulated by the small RNA MicF in a MarA-dependent way. These findings illustrate how broadly bacteria can mutate within a selective clinical setting, in this case resistance to carbapenems, by altering three porin genes and one regulatory gene.
Carbapenems are broad-spectrum β-lactam antibiotics used for the treatment of multi-drug resistant Gram-negative pathogens [1-3]. Carbapenem resistance most commonly arises through the acquisition of genes encoding carbapenemases, which hydrolyze carbapenems [3-5]. The other chief mechanism of carbapenem resistance in *E. coli* and other *Enterobacteriaceae* is decreased bacterial cell permeability due to loss or alteration of outer membrane porins F and/or C [1, 6-8].

The *marRAB* operon of *E. coli* encodes the MarR repressor, the transcriptional regulator MarA, and a putative small protein, MarB [9]. MarR represses transcription of *marRAB* by binding to *marO* and negatively controlling MarA-dependent expression of other genes in the regulon [10, 11]. Upon induction by a variety of compounds [12], or by mutation of *marR* or *marO*, the repressor is rendered inactive [10]. The resulting overexpression of MarA produces antibiotic resistance by increasing the expression of the major multi-drug efflux pump, AcrAB-TolC [13, 14] and down-regulating the outer membrane protein, OmpF via the sRNA MicF [15] [16]. In this study, a carbapenem-resistant, non-carbapenemase producing clinical isolate of *E. coli* from China (CH4) was investigated to determine the genetic basis for the carbapenem resistance phenotype.

PCR amplification and sequencing using primers “marR-for” (5’-ATTAGCGGCCGACGTCGAATTCAT) and “marR-rev” (5’-ATAGGATCCCTACGCTAGGTGAT) revealed numerous mutations in the *marR* ORF of strain CH4 and other clinical isolates from China (Table 1). We cloned ORFs containing the various *marR* mutations, using primers marR-clone-For and marR-clone-Rev [17], into...
expression vector pET-13a [18], where expression was controlled by the T7 promotor. Expression of T7 polymerase was induced from plasmid from plasmid pACT7-Spc [19] via IPTG in reporter strain SPC-106, a marO-lacZ fusion that contains a ΔmarR mutation [12]. Analysis of LacZ activity [11, 20] showed that the Gly42Arg mutation in the CH4 marR gene did not complement the ΔmarR mutation in this reporter strain (Figure 1) indicating that this mutation affected the activity of MarR.

We then complemented the marR mutation in CH4 by transforming the strain with pET-marR\textsubscript{wildtype} and pACT7-Spc or pAC-MarR\textsubscript{wt} [17]. MICs were determined. The data showed that both expression vectors produced similar decreases in resistance in strain CH4, which were not seen with an empty vector control (Table 1). We hypothesized that this effect was due to expression of \textit{ompF}; however, sequencing showed this gene to be inactivated by a partial deletion mutation. Upon extraction of outer membrane proteins from CH4 derivative strains (listed in Figure 2), we found a ~ 30 kDa protein newly expressed upon addition of wild type MarR (Figure 2). This protein was purified and processed for N-terminal sequencing, which revealed the protein to be YedS. The encoding gene, \textit{yedS}, is a previously described pseudogene which is untranslatable due to a large gap in the ORF in most sequenced strains. Sequence analysis of the \textit{yedS} gene in strain CH4 showed a complete and translatable gene (GenBank Accession number: JX392406).

Subsequent cloning of \textit{yedS}_{CH4} into pET13a via amplification with primers yed-nde-for (5\textendash-GCGCCATATGAAAAAGAAAATTTCTGG) and yed-bam-rev (5\textendash-ATAAGGATCCGAACTGGTAGACGATA) revealed it to be transcribed and translated into a similarly sized outer membrane protein in strain CH4 (Figure 2). When these plasmid-bearing strains were tested in MIC studies, decreased carbapenem resistance was observed in strains
CH4 and BL21DE3 (Invitrogen) (Table 1) indicating that YedS<sub>CH4</sub> is involved in carbapenem resistance.

To investigate the link of <i>yedS<sub>CH4</sub></i> transcription to MarA, we engineered a <i>yedS<sub>CH4</sub>-lacZ</i> promoter fusion plasmid using primers yedS-lac pro for (5’)-

**GCACCAATTGCCCCGAAAATTCAGAC** and yedS-lac pro rev (5’)-

**AGTCGGATCCTGTATTCCCTTGTA**) and reporter plasmid pRS415 [21]. This construct was transformed into lacZ- strains from the Keio collection [22] [23] containing mutations in either <i>marR</i> or <i>marRA</i> (Table 2). When these strains were grown to late log phase at temperatures of 37°C, we found expression of the <i>yedS<sub>CH4</sub></i> promoter was ~30% in the <i>marR</i> strain compared to its wild type parent. However when the <i>marR</i> strain also contained a <i>marA</i> deletion, transcription of <i>yedS<sub>CH4</sub>-lacZ</i> was equal to the parental strain. Suspecting that this relationship was due to the MarA regulated <i>micF</i>, we transduced [24] a <i>micF::Cm</i> mutation into these strains, and observed a restored transcription of <i>yedS<sub>CH4</sub>-lacZ</i> in all strains(Table 2). Thus the <i>mar</i> operon controls expression of <i>yedS<sub>CH4</sub></i> via the sRNA <i>micF</i>.

Our findings implicate the outer membrane protein YedS<sub>CH4</sub> in carbapenem sensitivity/resistance. We hypothesize that the maintenance of a functional YedS in strain CH4, is an evolutionary response to the lack of functional OmpF and OmpC. Additionally, the presence of this carbapenem portal presents a selective pressure for this strain to maintain its novel <i>marR</i> mutation, downregulating <i>yedS<sub>CH4</sub></i> expression via MicF, and producing resistance to carbapenems. In the absence of carbapenemase, selection may occur for <i>mar</i> mutants which will be resistant to a greater spectrum of antibiotics and potentially have greater virulence [25, 26] than parental strains containing functional <i>marR</i> genes. Our findings suggest how this uniquely selective environment could affect genetic fluidity of the bacterial cell that seeks to survive in
response to different insults. The isolate described here has mutated two of its porins, enabled a pseudogene to be expressed, and derepressed the \textit{marRAB} operon, sufficient to produce a drug resistant strain. The order in which these mutations occurred is not known; however, the accumulation of so many mutations in a single isolate is a clear display of bacterial adaptation.

Acknowledgements

We thank Biomerieux for providing the E-tests used in this work.


Table 1. Effects of complementation of wild-type MarR and YedS\textsubscript{CH4} on carbapenem susceptibility in clinical and laboratory strains of \textit{E. coli}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation in \textit{marR}</th>
<th>MIC\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH4</td>
<td>Gln42Arg, Gly103Ser, Tyr137His</td>
<td>&gt;32 32 &gt;32</td>
</tr>
<tr>
<td>CH4/pACT7/pET13a</td>
<td>&gt;32 32 &gt;32</td>
<td></td>
</tr>
<tr>
<td>CH4/pACT7/p\textit{marR}_{wildtype}</td>
<td>0.25 0.38 0.25</td>
<td></td>
</tr>
<tr>
<td>CH4/pACT7/pyedS\textsubscript{CH4}</td>
<td>1.5 1.5 4.0</td>
<td></td>
</tr>
<tr>
<td>CH4/pAC-MarRwt</td>
<td>0.25 0.38 0.25</td>
<td></td>
</tr>
<tr>
<td>BL21DE3/pET13a</td>
<td>Wild type \textit{marR}</td>
<td>0.047 0.023\textsuperscript{b} 0.047</td>
</tr>
<tr>
<td>BL21DE3/pyedS\textsubscript{CH4}</td>
<td>0.004 0.023\textsuperscript{b} 0.004</td>
<td></td>
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</tbody>
</table>

\textsuperscript{a} MICs were determined using Etest (bioMérieux). CH4 cells were cultured on LB-agar containing kanamycin (800 \(\mu\)g/ml) and specinomycin (200 \(\mu\)g/ml) when carrying pET13a plasmids and pACT7 plasmids respectively. BL21DE3 cells were cultured on agar containing kanamycin (50 \(\mu\)g/ml) when carrying the pET13a plasmid. All cultures were induced with IPTG (0.5mM).

\textsuperscript{b} Meropenem MICs were not affected by overexpression of YedS in BL21DE3, most likely due to other factors in this strain which affect susceptibility to this carbapenem.
Table 2: Effects of *marR*, *marA*, and *micF* mutations on *yedS*$_{CH4}$ promoter$^a$

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>micF</em>+</th>
<th><em>micF</em>::Cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW25113</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CR1000 (<em>ΔmarR</em>)</td>
<td>0.29 ± 0.13</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>CR2000 (<em>ΔmarRA</em>)</td>
<td>0.96 ± 0.07</td>
<td>1.29 ± 0.09</td>
</tr>
</tbody>
</table>

$^a$ Data represent β-galactosidase values relative to the wild type strain BW25113 containing plasmid pRS415-*yedS*$_{CH4}$-*lacZ*, grown under the same conditions ± the standard error of the mean. All strains were grown in LB-broth supplemented with ampicillin (100 μg ml$^{-1}$) to maintain carriage of pRS415-*yedS*$_{CH4}$-*lacZ*. 
Figure 1

**Reporter gene assay for MarR function.**

Reporter strain SPC105ΔmarR, which lacks the marR gene, carries a chromosomal Pmar::lacZ transcriptional fusion. Results were expressed as a percentage of transcription activity for the control (SPC105ΔmarR bearing pACT7 and pET13a without the insert) and are the means and standard deviations of results from at least three replicated assays. All assays were performed as described [20]. The origins of the cloned marR genes were as follows: bar 1, none; bar 2, wild-type marR; bar 3, marR gene encoding the Lys62Arg, Gly103Ser and Tyr137His mutations; bar 4, marR gene encoding the Gly103Ser and Tyr137His mutations; bar 5, marR gene encoding Ala53Glu, Gly103Ser and Tyr137His; bar 6 mutations, marRCH4 gene encoding Gln42Arg, Gly103Ser and Tyr137His mutations.
Urea SDS-PAGE analysis of outer membrane proteins.
Outer membrane proteins were purified and subjected to gel electrophoresis as described in [27]. The arrow to the left denotes the migration of the 37kDa molecular weight marker. Each lane was loaded with 5 µg of total outer membrane protein.