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Involvement of MarR and YedS in Carbapenem Resistance in a Clinical Isolate of *Escherichia coli* from China

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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 nontranslated 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 multidrug-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 *Escherichia coli* and other *Enterobacteriaceae* is decreased bacterial cell permeability due to loss or alteration of the 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 multidrug efflux pump AcrAB-TolC (13, 14) and downregulating the outer membrane protein OmpF via the small RNA (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 the primers marR-for (5′-ATTAGCGGCCGCATCGGTCAATTCAT) and marR-rev (5′-ATAGGATCCTTACGGCTGCGGATGTA) revealed numerous mutations in the *marR* open reading frame (ORF) of strain CH4 and other clinical isolates from China (Table 1). We cloned ORFs containing the various *marR* mutations, using the primers marR-clone-For and marR-clone-Rev (17), into expression vector pET-13a (18), for which expression was controlled by the T7 promoter. Expression of T7 polymerase was induced from plasmid pACT7-Spc (19) via isopropyl-β-D-thiogalactopyranoside (IPTG) in the reporter strain SPC-106, a *marO-lacZ* fusion that contains a Δ*marR* mutation (12). Analysis of LacZ activity (11, 20) showed that the Gly42Arg mutation in the CH4 *marR* gene did not complement the Δ*marR* mutation in this reporter strain (Fig. 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$_{wildtype}$ and pACT7-Spc or pAC-MarRwt (17). MICs were determined. The data showed that the two expression vectors produced similar decreases in resistance in strain CH4 that were not seen with an empty vector con-

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TABLE 1 Effects of complementation of wild-type MarR and YedS$_{CH4}$ on carbapenem susceptibility in clinical and laboratory strains of *E. coli*

Strain	Mutation(s) in *marR*	MIC (μg/ml)[a]		
		Imipenem	Meropenem	Ertapenem
CH4	Gln42Arg, Gly103Ser, Tyr137His	>32	32	>32
CH4/pACT7/pET13a		>32	32	>32
CH4/pACT7/pmarR$_{wildtype}$		0.25	0.38	0.25
CH4/pACT7/pyedS$_{CH4}$		1.5	1.5	4.0
CH4/pAC-MarRwt		0.25	0.38	0.25
BL21(DE3)/pET13a	Wild-type *marR*	0.047	0.023[b]	0.047
BL21(DE3)/pyedS$_{CH4}$		0.004	0.023[b]	0.004

[a] MICs were determined using Etest (bioMérieux). CH4 cells were cultured on LB agar containing kanamycin (800 μg/ml) and spectinomycin (200 μg/ml) when carrying pET13a plasmids and pACT7 plasmids, respectively. BL21(DE3) cells were cultured on agar containing kanamycin (50 μg/ml) when carrying the pET13a plasmid. All cultures were induced with IPTG (0.5 mM).
[b] Meropenem MICs were not affected by overexpression of YedS in BL21(DE3), most likely due to other factors in this strain which affect susceptibility to this carbapenem.

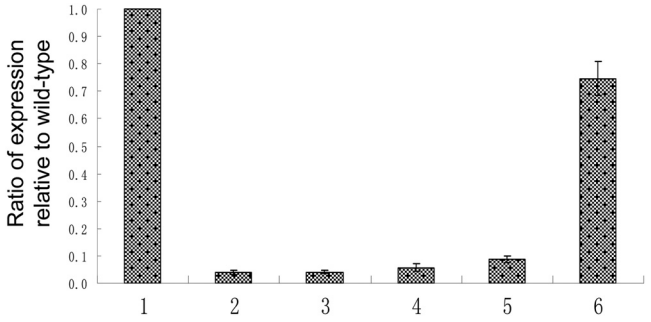

FIG 1 Reporter gene assay for MarR function. The reporter strain SPC105Δ*marR*, which lacks the *marR* gene, carries a chromosomal $Pmar_{II}$::*lacZ* transcriptional fusion. Results are expressed as percentages of transcription activity of the control (SPC105Δ*marR* bearing pACT7 and pET13a without the insert) and are the means and standard deviations of results from at least three replicated assays. All assays were performed as described previously (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 the Ala53Glu, Gly103Ser, and Tyr137His mutations; bar 6, $marR_{CH4}$ gene encoding the Gln42Arg, Gly103Ser, and Tyr137His mutations.

trol (Table 1). We hypothesized that this effect was due to expression of *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 Fig. 2), we found an ~30-kDa protein newly expressed upon addition of wild-type MarR (Fig. 2). This protein was purified and processed for N-terminal sequencing, which revealed the protein to be YedS. The encoding gene, *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 *yedS* gene in strain CH4 showed a complete and translatable gene.

Subsequent cloning of $yedS_{CH4}$ into pET13a via amplification with primers yed-nde-for (5′-GCGCCATATGAAAAGAAAAGTTCTGG) and yed-bam-rev (5′-ATAAGGATCCGAACTGGTAGACGATA) revealed it to be transcribed and translated into a similarly sized outer membrane protein in strain CH4 (Fig. 2). When these plasmid-bearing strains were tested in MIC studies, decreased carbapenem resistance was observed in strains CH4 and BL21(DE3) (Invitrogen) (Table 1), indicating that $YedS_{CH4}$ is involved in carbapenem resistance.

To investigate the link of $yedS_{CH4}$ transcription to MarA, we engineered a $yedS_{CH4}$-*lacZ* promoter fusion plasmid using primers yedS-lac pro for (5′-GCACCAATTGCCCGGAAAATTCAGAC) and yedS-lac pro rev (5′-AGTCGGATCCTGTATTCCCTTGTGA) and reporter plasmid pRS415 (21). This construct was

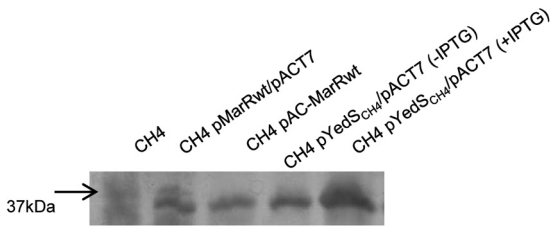

FIG 2 Urea SDS-PAGE analysis of outer membrane proteins. Outer membrane proteins were purified and subjected to gel electrophoresis as described previously (27). The arrow to the left denotes the migration of the 37-kDa molecular weight marker. Each lane was loaded with 5 μg of total outer membrane protein.

TABLE 2 Effects of *marR*, *marA*, and *micF* mutations on the $yedS_{CH4}$ promoter[a]

Strain	Relative β-galactosidase value	
	micF$^+$	*micF*::Cm
BW25113	1	1
CR1000 (Δ*marR*)	0.29 ± 0.13	1.01 ± 0.07
CR2000 (Δ*marRA*)	0.96 ± 0.07	1.29 ± 0.09

[a] Data represent β-galactosidase values relative to that of the wild-type strain BW25113 containing plasmid pRS415-$yedS_{CH4}$-lacZ, grown under the same conditions, and 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.

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

Our findings implicate the outer membrane protein $YedS_{CH4}$ 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 *marR* mutation, down-regulating $yedS_{CH4}$ expression via MicF and producing resistance to carbapenems. In the absence of carbapenemase, selection may occur for *mar* mutants which will be resistant to a greater spectrum of antibiotics and potentially have greater virulence (25, 26) than parental strains containing functional *marR* genes. Our findings suggest how this uniquely selective environment may 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 *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.

Nucleotide sequence accession number. The sequence of the *yedS* gene in strain CH4 was deposited in GenBank under accession number JX392406.

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