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## ORIGINAL ARTICLE

# Role of the AraC–XylS family regulator YdeO in multi-drug resistance of *Escherichia coli*

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Multi-drug efflux pumps contribute to the resistance of *Escherichia coli* to many antibiotics and biocides. In this study, we report that the AraC–XylS family regulator YdeO increases the multi-drug resistance of *E. coli* through activation of the MdtEF efflux pump. Screening of random fragments of genomic DNA for their ability to increase β-lactam resistance led to the isolation of a plasmid containing *ydeO*, which codes for the regulator of acid resistance. When overexpressed, *ydeO* significantly increased the resistance of the *E. coli* strain to oxacillin, cloxacillin, nafcillin, erythromycin, rhodamine 6G and sodium dodecyl sulfate. The increase in drug resistance caused by *ydeO* overexpression was completely suppressed by deleting the multifunctional outer membrane channel gene *tolC*. TolC interacts with different drug efflux pumps. Quantitative real-time PCR showed that YdeO activated only *mdtEF* expression and none of the other drug efflux pumps in *E. coli*. Deletion of *mdtEF* completely suppressed the YdeO-mediated multi-drug resistance. YdeO enhances the MdtEF-dependent drug efflux activity in *E. coli*. Our results indicate that the YdeO regulator, in addition to its role in acid resistance, increases the multi-drug resistance of *E. coli* by activating the MdtEF multi-drug efflux pump.

## INTRODUCTION

Multi-drug efflux pumps cause serious problems in cancer chemotherapy and in the treatment of bacterial infections. Bacterial drug resistance is often associated with multi-drug efflux pumps that decrease drug accumulation in the cell.[1,2] Bacterial multi-drug efflux pumps are classified into five families on the basis of sequence similarity: major facilitator, resistance-nodulation-cell division (RND), small multi-drug resistance, multidrug and toxic compound extrusion, and ATP-binding cassette.[3–5] Of these, RND family efflux pumps play major roles in both intrinsic and elevated resistance of Gram-negative bacteria to a wide range of compounds, including β-lactams.[1,6–12] RND efflux pumps require two other proteins to function: a membrane fusion protein and an outer membrane protein. Many drug efflux pumps in *Escherichia coli* need TolC to function.[12–15] TolC is responsible for resistance to various antibiotics, including β-lactams,[12] quinolones[16] and macrolides.[17] Bacterial genome sequencing enables us to trace drug-resistance genes.[18–20] There are many putative and proven drug efflux pumps in the *E. coli* genome, and we have identified earlier 20 functional drug efflux pumps.[9,20] As many such efflux pumps have overlapping substrate spectra,[9] it is intriguing that bacteria, with their economically organized genomes, harbor such large sets of multi-drug efflux genes.

The key to understanding how bacteria utilize these multiple efflux pumps lies in the regulation of pump expression. The currently available data show that multi-drug efflux pumps are often expressed under precise and elaborate transcriptional control.[21–24] Expression of *acrAB*, which encodes the major AcrAB efflux pump, is subject to multiple levels of regulation. In *E. coli*, it is modulated locally by the repressor AcrR[25] and AcrS.[26] At a more global level, it is modulated by stress conditions and by global regulators, such as MarA, SoxS and Rob.[27,28] These examples illustrate the complexity and diversity of the mechanisms regulating bacterial multi-drug efflux pumps.

Stomach acid (pH ⩽ 2) kills most bacteria;[29] however, *E. coli* survives this acidity.[30,31] The acid resistance appears to contribute to the low infectious dose of pathogenic *E. coli*, and aids in the gastric passage of commensal strains.[32,33] One of the most efficient acid resistance systems in *E. coli*, the Gad system, is based on the coordinated action of two isoforms of glutamate decarboxylase (GadA and GadB) and of a specific glutamate/γ-aminobutyrate (GABA) antiporter (GadC).[34,35] The *gadA/BC* genes, activated in response to acid stress, are subject to complex circuits of regulation involving the AraC–XylS family regulator YdeO.[36] In this study, we demonstrate that YdeO contributes to the multi-drug resistance as well as the acid resistance of *E. coli*. The results suggest a role of YdeO in the multi-drug resistance of *E. coli*.

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## MATERIALS AND METHODS

### Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *E. coli* strains were derived from the wild-type strain MG1655.[37] Phage P1-mediated transductions were performed as described earlier.[38] Bacterial strains were grown at 37 °C in Luria–Bertani (LB) broth.[39] Cells were collected for total RNA extraction when the cultures reached an optical density of 0.6 at 600 nm.

### Screening for positive regulators of multi-drug resistance

DNA manipulation generally followed standard practice.[39] A genomic library was developed by partial *Sau*3AI digestion of the chromosomal DNA as follows. Chromosomal DNA prepared from an overnight culture of the wild-type strain MG1655[37] was digested with *Sau*3AI (1 U μl$^{-1}$) for 15, 20, 30 and 40 min. The digested DNA was separated on a 0.8% agarose gel, and fragments approximately 0.5–3 kb in size were purified and ligated into the *Bam*HI site of vector pHSG398 (Takara Bio Inc., Otsu, Japan). The ligation products were transformed into *E. coli* DH5α[39] to select chloramphenicol-resistant transformants. Plasmid DNA was prepared from a pool of 16,000 transformants and used to transform the *acrB* deletion strain NKE96. Cells were plated on LB agar medium[39] containing 15 μg ml$^{-1}$ chloramphenicol and inhibitory concentrations of various drugs.

### Plasmid construction

The *ydeO* gene was amplified from MG1655 genomic DNA using the primers ydeO-F_*Bam*HI and ydeO-R_*Sal*I listed in Table 2, which introduced *Bam*HI and *Sal*I sites at the ends of the amplified fragment. This PCR product was cloned between the *Bam*HI and *Sal*I sites of the vector pHSG398 (Takara Bio Inc.) to produce the plasmid p*ydeO*.

### Construction of gene deletion mutants

Gene deletion was performed according to the method of Datsenko and Wanner, with recombination between short homologous DNA regions catalyzed by phage λ Red recombinase.[40] A curable expression plasmid encoding Red recombinase (pKD46) was introduced into the MG1655 strain. The chloramphenicol resistance gene *cat* or the kanamycin resistance gene *kan*, flanked by Flp recognition target sites, was amplified by PCR using the primers listed in Table 2. The plasmid pKD3 or pKD4 was used as a template. This PCR product was used to transform the MG1655 strain expressing Red recombinase, and recombinant clones were isolated as chloramphenicol- or kanamycin-resistant colonies. The pKD46 vector was eliminated by incubating at a non-permissive temperature (37 °C), as confirmed by the loss of ampicillin resistance. The chromosomal structure of the mutated loci was verified by PCR, as described earlier,[40] and by Southern hybridization using probes specific for (i) the antibiotic resistance genes used during the construction of chromosomal deletions and (ii) sequences flanking the inactivated loci. The deletions were then transferred to the wild-type MG1655 strain by P1 transduction. The *cat* and *kan* genes were eliminated using the plasmid pCP20, as described earlier.[40]

### Determination of the MICs for toxic compounds

The antibacterial activities of different agents were determined on Luria agar (1% tryptone, 0.5% yeast extract and 0.5% NaCl) plates containing various concentrations of the compounds (Sigma-Aldrich, St Louis, MO, USA) listed in Table 3. The agar plates were prepared by the two-fold agar dilution technique, as described earlier.[41] The MIC was defined as the lowest concentration of a compound that inhibited cell growth. To determine the MICs, bacteria were grown in LB broth at 37 °C overnight and diluted in the same medium. Then, the organisms were tested at a final inoculum size of 10$^4$ cfu μl$^{-1}$ using a multipoint inoculator (Sakuma Seisakusyo, Tokyo, Japan), and were incubated at 37 °C for 20 h.

### RNA extraction

Total RNA was isolated from bacterial cultures using the RNeasy Protect Bacteria Mini Kit (Qiagen, Hilden, Germany) and RNase-Free DNase (Qiagen), as described earlier.[42] The total RNA was isolated from exponential-phase cultures of Δ*acrB*/vector (NKE154) and Δ*acrB*/*pydeO* (NKE169). The absence of genomic DNA from the DNase-treated RNA samples was confirmed by both non-denaturing agarose electrophoresis gels and PCR with primers against target genomic DNA. The RNA concentration was then determined spectrophotometrically.[39]

### Determination of specific transcript levels by quantitative real-time PCR following reverse transcription (RT)

Bulk cDNA samples were synthesized from total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA, USA) and random hexamers, as described earlier.[43,44] The specific primer pairs listed in Table 2 were designed using ABI PRISM Primer Express software (Applied Biosystems). *rrsA* of 16S rRNA was chosen as the normalizing gene.[45] Real-time PCR was

### Table 1 *E. coli* strains and plasmids used in this study

| Strains or plasmids | Original names | Characteristics | Sources or references |
|---|---|---|---|
| *Strains as in text* | | | |
| WT | MG1655 | Wild type | [37] |
| Δ*acrB* | NKE 96 | Δ*acrB* | Present study |
| Δ*acrB*/vector | NKE 154 | Δ*acrB*/pHSG398 | Present study |
| Δ*acrB*/*pydeO* | NKE 169 | Δ*acrB*/*pydeO* | Present study |
| Δ*acrB* *tolC* | NKE 128 | Δ*acrB* Δ*tolC* | Present study |
| Δ*acrB* *tolC*/vector | NKE 160 | Δ*acrB* Δ*tolC*/pHSG398 | Present study |
| Δ*acrB* *tolC*/*pydeO* | NKE 174 | Δ*acrB* Δ*tolC*/*pydeO* | Present study |
| Δ*acrB* *mdtEF* | NKE 139 | Δ*acrB* Δ*mdtEF*::Km$^R$ | Present study |
| Δ*acrB* *mdtEF*/vector | NKE 176 | Δ*acrB* Δ*mdtEF*::Km$^R$/pHSG398 | Present study |
| Δ*acrB* *mdtEF*/*pydeO* | NKE 180 | Δ*acrB* Δ*mdtEF*::Km$^R$/*pydeO* | Present study |
| | | | |
| *Plasmids* | | | |
| pKD46 | | Red recombinase expression plasmid, Ap$^R$ | [40] |
| pKD3 | | rep$_{R6K\gamma[\rho]}$Ap$^R$FRT Cm$^R$FRT | [40] |
| pKD4 | | rep$_{R6K\gamma[\rho]}$Ap$^R$FRT Km$^R$FRT | [40] |
| pCP20 | | rep$_{pSC101}$$^{ts}$ Ap$^R$ Cm$^R$ *cI857*λP$_R$*flp* | [40] |
| pHSG398 | | rep$_{pMBI}$ Cm$^R$ | Takara Bio Inc. |
| p*ydeO* | | *ydeO* gene cloned into pHSG398, Cm$^R$ | Present study |

## Table 2 Primers used in the present study

| Primers | Sequences (5′–3′) |
| --- | --- |
| *For gene deletion* | |
| *tolC*-P1 | ACTGGTGCCGGGCTATCAGGCGCATAACCATCAGCAATAGGTGTAGGCTGGAGCTGCTTC |
| *tolC*-P2 | TTACAGTTTGATCGCGCTAAATACTGCTTCACCACAAGGACATATGAATATCCTCCTTAG |
| *acrB*-P1 | AAAAAGGCCGCTTACGCGGCCTTAGTGATTACACGTTGTAGTGTAGGCTGGAGCTGCTTC |
| *acrB*-P2 | GAACAGTCCAAGTCTTAACTTAAACAGGAGCCGTTAAGACCATATGAATATCCTCCTTAG |
| *mdtE*-P1 | TTAAAGAACCGTTATTTCTCAAGAATTTTCAGGGACTAAAGTGTAGGCTGGAGCTGCTTC |
| *mdtF*-P2 | AGGCTGAACCTTCATGTTCAACCTTACTCTCATTTACACGCATATGAATATCCTCCTTAG |
| *For gene cloning* | |
| *ydeO*-F_BamHI | CGCGGATCCAAACAAACAGCAAATTATAAA |
| *ydeO*-R_SalI | CGCGTCGACTTTCAATAAATATATCGCCTA |
| *For quantitative* PCR | |
| *rrsA*-F | CGGTGGAGCATGTGGTTTAA |
| *rrsA*-R | GAAAACTTCCGTGGATGTCAAGA |
| *acrA*-F | GTCTATCACCCTACGCGCTATCTT |
| *acrA*-R | GCGCGCACGAACATACC |
| *acrD*-F | GTACCCTGGCGGATTTTTTCATT |
| *acrD*-R | CGGTCACTCGCACATTCG |
| *acrE*-F | CGTGATTGCCGCAAAAGC |
| *acrE*-R | TTGGCGCAGTGACTTTGGTA |
| *bcr*-F | TGTTTTTCTGTTCGTGATGACCAT |
| *bcr*-R | GGAACATATTTAACGCGCCAAT |
| *cusB*-F | CGCTTACCGTGGGCGATA |
| *cusB*-R | TTCCACCCAGTCAGGAATGG |
| *emrA*-F | GCGAATATTGAGGTGCAGAAAA |
| *emrA*-R | GGCACACGGCGGTTGTA |
| *emrD*-F | GTGGATCCCCGACTGGTTT |
| *emrD*-R | CCCGGCACCGAAAAAGA |
| *emrE*-F | GGTATTGTCCTGATTAGCTTACTGTCAT |
| *emrE*-R | GCACAAATCAACATCATGCCTATAA |
| *emrK*-F | GCGCTTAAACGTACGGATATTAAGA |
| *emrK*-R | ACTGTTTCGCCGACCTGAAC |
| *fsr*-F | TGGTGTTGGCGCAAATCA |
| *fsr*-R | TCGTCGCTTTGGGTTTTCC |
| *macA*-F | CGGTGATTGCCGCACAA |
| *macA*-R | TTACCAGCATGGCGCTCAT |
| *mdfA*-F | CTTGCTGTTAGCGCGTCTGA |
| *mdfA*-R | GCCAGCCGCCCATAATAAT |
| *mdtA*-F | CGCCGTAGAACAGGCAGTTC |
| *mdtA*-R | TGCGCACCGTAACGGTATTA |
| *mdtE*-F | CCCCCGGTTCGGTCAA |
| *mdtE*-R | GGACGTATCTCGGCAACTTCAT |
| *mdtF*-F | TTACCGTCAGCGCTACCTATCC |
| *mdtF*-R | GCCATCAAGCCCATTCATATTT |
| *mdtG*-F | CGGTATTGTCTTCAGCATTACATTTT |
| *mdtG*-R | GGCGAGTCCACCCCAAA |
| *mdtH*-F | TTTTCACCCTGATTTGTCTGTTTTAT |
| *mdtH*-R | CAGCGAAGCACTTAAGGTTTCA |
| *mdtJ*-F | TGATGAAAATTGCCGGGTTAA |
| *mdtJ*-R | CGCTTTACGGGTACCTGATTTTA |
| *mdtK*-F | CCGGTTATCGCGCAATTAAAT |
| *mdtK*-R | GAAACCTTGTCGCACCTGATG |
| *mdtL*-F | TATCCCGCCGGGATTGATAT |
| *mdtL*-R | CGCTTCGCTGGCATTGA |
| *mdtM*-F | CGTGATTTTAATGCCGATGTCA |
| *mdtM*-R | GCCATACCGCCAGCAAGAT |
| *tolC*-F | CCGGGGATTTCTGACACCTCTT |
| *tolC*-R | TTTGTTCTGGCCCATATTGCT |

**Table 3 Susceptibility of *E. coli* strains to β-lactams and toxic compounds**

| | MIC (µg/ml) | | | | | |
|---|---|---|---|---|---|---|
| *Strains* | OXA | MCIPC | NAF | ERM | R6G | SDS |
| WT | 256 | >512 | 512 | 128 | >512 | >512 |
| ΔacrB | 0.5 | 1 | 2 | 4 | 2 | 64 |
| ΔacrB/vector | 0.5 | 1 | 2 | 4 | 2 | 64 |
| ΔacrB/pydeO | **16** | **64** | **32** | **64** | **256** | **>512** |
| ΔacrB tolC | 0.5 | 0.25 | 1 | 2 | 2 | 16 |
| ΔacrB tolC/vector | 0.5 | 0.25 | 1 | 2 | 2 | 16 |
| ΔacrB tolC/pydeO | 0.5 | 0.25 | 1 | 2 | 2 | 16 |
| ΔacrB mdtEF | 0.5 | 1 | 2 | 4 | 2 | 64 |
| ΔacrB mdtEF/vector | 0.5 | 1 | 2 | 4 | 2 | 64 |
| ΔacrB mdtEF/pydeO | 0.5 | 1 | 2 | 4 | 2 | 64 |

Abbreviations: ERM, erythromycin; MCIPC, cloxacillin; NAF, nafcillin; OXA, oxacillin; R6G, rhodamine 6G; SDS, sodium dodecyl sulfate.
Values in bold face are larger than those of a corresponding parental strain haboring the pHSG398 vector. MIC determinations were repeated at least three times.

performed with each specific primer pair using SYBR Green PCR Master Mix (Applied Biosystems). The reactions were run on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems); the fluorescence signal due to SYBR Green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle. The expression levels of drug efflux pump genes and *tolC* in Δ*acrB*/*pydeO* (NKE169) were compared with those in Δ*acrB*/vector (NKE154).

### Acid resistance assay

A single colony of an *E. coli* strain harboring plasmid was inoculated into 1 ml of LB broth containing chloramphenicol and grown overnight at 37 °C. The LB broth (20 ml) was inoculated with 0.1 ml of the overnight culture and grown at 37 °C. When the cultures reached a cell density of $2 \times 10^8$ cfu ml$^{-1}$, 50 µl of the culture was transferred to 2 ml of phosphate-buffered saline (PBS; pH 7.2) and to 2 ml of warmed LB broth (pH 2.5, adjusted with HCl). The cfu ml$^{-1}$ in PBS was determined by plating serial dilutions in PBS buffer (pH 7.2) on LB agar and using these as initial cell populations. The LB broth (pH 2.5) inoculated with *E. coli* was incubated at 37 °C for 1 h, and the cfu ml$^{-1}$ in the LB broth (pH 2.5) was determined as described above and used as the final cell population. The percentage acid survival was then calculated as the number of cfu ml$^{-1}$ remaining after acid treatment divided by the initial cfu ml$^{-1}$ at time zero. Each experiment was performed in triplicate. The percentage survival values were converted to logarithmic values ($\log_{10} x$, where $x$ equals the percentage survival) for the calculation of geometric means and standard errors (s.e.).

### Drug efflux assay

The drug efflux activities of *E. coli* cells were measured using cells preloaded with rhodamine 6G. The exponential cultures of *E. coli* cells were harvested and washed twice with 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM MgSO$_4$. For maximal accumulation of the fluorophore, the cells (optical density of 1.0 at 600 nm) were incubated with 1 µM rhodamine 6G and 40 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at 37 °C for 1 h. The cells were then centrifuged, resuspended in the same medium with the addition of 25 mM glucose as an energy source, and subjected to fluorescence measurement. The fluorescence of the compound was continuously monitored using a Hitachi model F-2000 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). Rhodamine 6G transport was measured with excitation at 529 nm and emission at 553 nm.

## RESULTS
### Overexpression of *ydeO* increases resistance to oxacillin

The expression of multi-drug efflux genes is often regulated in a complex manner, as described in the Introduction section. We there-

fore screened the genomic library of the *E. coli* for genes that increased multi-drug resistance levels in this organism. We screened a host strain lacking a functional *acrB* gene in order to identify regulatory elements involved in the expression of other multi-drug resistance systems. The library was developed from the chromosomal DNA of the MG1655 strain, and then, the recombinant plasmids were transformed into the Δ*acrB* strain NKE96 as described in the Materials and methods section. In one experiment, we found a 32-fold increase in oxacillin MIC against the transformant (data not shown). Introduction of the plasmid isolated from this strain into fresh Δ*acrB* cells resulted in the same oxacillin resistance phenotype: the MIC increased 32-fold over the recipient strain (data not shown).

Sequencing of the plasmid revealed an insertion containing the complete coding sequence of *ydeO* and a partial sequence of *yneN*. YdeO is the AraC–XylS family regulator that controls genes involved in acid resistance, such as glutamate decarboxylase genes (*gadA* and *gadB*) and a specific glutamate/GABA antiporter (*gadC*).[34,36] It seemed likely that overexpressed YdeO caused the transcriptional activation of genes involved in oxacillin resistance in the cells carrying this plasmid.

Full-length wild-type *ydeO* was cloned into the pHSG398 vector to obtain p*ydeO* (Table 1). Oxacillin MICs for NKE96 cells harboring p*ydeO* were 32 times higher (16 versus 0.5 µg ml$^{-1}$) than that for cells harboring the pHSG398 vector (Table 3), suggesting that the YdeO regulator produced by this plasmid conferred oxacillin resistance on *E. coli*. Further experiments were therefore carried out with p*ydeO*.

### Overexpression of *ydeO* increases resistance to β-lactams, erythromycin, rhodamine 6G and sodium dodecyl sulfate (SDS)

Our results showed that overexpression of *ydeO* increased *E. coli* resistance to oxacillin, a β-lactam antibiotic. We therefore investigated the effect of *ydeO* overexpression on the susceptibility of *E. coli* to other β-lactams. p*ydeO* also increased the resistance of Δ*acrB* cells to cloxacillin and nafcillin (Table 3). Various other drugs were tested, including common substrates of multi-drug efflux pumps, and we found that p*ydeO* increased the resistance of the Δ*acrB* strain to erythromycin, rhodamine 6G and SDS (Table 3). These results indicate that the overproduced YdeO regulator induces the multi-drug resistance of *E. coli*.

### Effect of *tolC* deletion on multi-drug resistance modulated by the YdeO regulator

The results described above indicate that the expression of a multi-drug efflux pump may be induced by overexpression of *ydeO*. In order to determine whether YdeO-mediated multi-drug resistance is attributable to TolC-dependent drug efflux pump(s), we investigated the effect of *tolC* deletion on drug resistance in cells overexpressing *ydeO*. Deletion of *tolC* from the Δ*acrB* strain increased susceptibility to many antimicrobial agents and chemical compounds, including cloxacillin, nafcillin, erythromycin and SDS (Table 3), which is in good agreement with earlier reports.[12] The *tolC* deletion completely inhibited YdeO-mediated multi-drug resistance (Table 3). This result indicates that YdeO-mediated multi-drug resistance is attributable to increased expression of a TolC-dependent drug efflux pump.

### Determination of the amounts of drug exporter transcripts by quantitative real-time RT-PCR (qRT-PCR)

In order to determine which drug efflux pump shows increased expression when *ydeO* is overexpressed, we used qRT-PCR to investigate changes in the amounts of drug exporter gene mRNAs. The results are shown in Figure 1. The expression levels of *mdtE* and *mdtF*
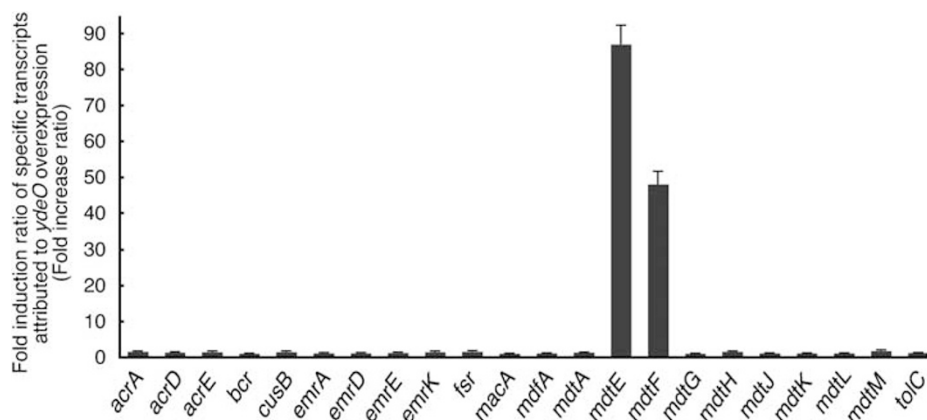
**Figure 1** YdeO activates the expression of *mdtEF* multi-drug efflux genes. The amount of transcript was determined by quantitative real-time PCR as described in the Materials and methods section. The fold increase ratio was calculated by dividing the expression level of the gene in the Δ*acrB*/p*ydeO* strain by that in the Δ*acrB*/vector strain. Experiments were performed in triplicate and the data are represented as mean ± s.d.

were significantly increased (more than 10-fold in comparison with basal levels) by *ydeO* overexpression: 87- and 48-fold increases were observed for *mdtE* and *mdtF*, respectively. Overexpression of *ydeO* did not increase the expression of other drug exporter gene transcripts (Figure 1).

### Effects of deletion of the MdtEF drug efflux pump on YdeO-mediated multi-drug resistance

To determine whether the multi-drug resistance mediated by *ydeO* overexpression is because of increased expression of *mdtEF*, we investigated the effects of deleting these genes on drug resistance levels in Δ*acrB*/vector and Δ*acrB*/p*ydeO* (Table 3). When *mdtEF* was deleted from the Δ*acrB* strain, there was no change in drug resistance in the resulting strains. In the Δ*mdtEF acrB* strain, overexpression of *ydeO* conferred no drug resistance (Table 3). Together, these data indicate that the multi-drug resistance conferred by the YdeO regulator is because of the increased expression of *mdtEF* multi-drug efflux genes.

### Effects of YdeO and MdtEF on the acid resistance of *E. coli*

It was reported that YdeO activates the expression of genes involved in acid resistance.[36] In this study, we found that YdeO induces the expression of *mdtEF* multi-drug efflux genes. To test whether MdtEF contributes to acid resistance modulated by YdeO, we measured survival percentage of the wild-type strain and the Δ*mdtEF* mutant harboring vector (pHSG398) or p*ydeO* at pH 2.5 (Figure 2). Overexpression of *ydeO* enhances the acid resistance of both the wild-type strain and the Δ*mdtEF* mutant (Figure 2), indicating that the MdtEF multi-drug efflux pump is not essential for YdeO-induced acid resistance.

### YdeO enhances the MdtEF-dependent drug efflux activity of *E. coli*

In this study, we found that YdeO activates the expression of *mdtEF* multi-drug efflux genes and drug resistance to *E. coli* cells. *E. coli* cells with a plasmid carrying the *ydeO* gene became resistant to rhodamine 6G, a toxic dye. This compound can be detected by its fluorescence. To determine whether the *ydeO*-induced drug resistance is because of efflux of this compound from the cells, we measured the efflux activity of rhodamine 6G from the cells. As shown in Figure 3, rapid efflux of rhodamine 6G from *E. coli* Δ*acrB*/p*ydeO* cells was observed as an increase in fluorescence. However, no significant efflux was observed from Δ*acrB* and Δ*acrB mdtEF*/p*ydeO* cells (Figure 3). These results indicate that the YdeO-induced multi-drug resistance is because of the
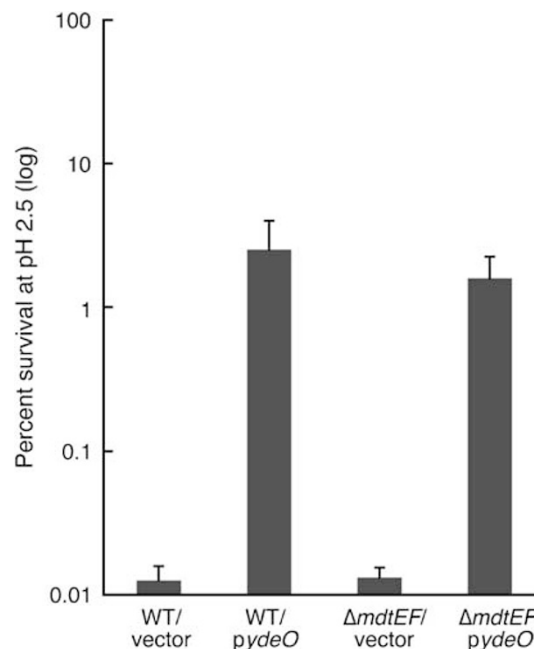


**Figure 2** Effect of YdeO and MdtEF on acid resistance of *E. coli*. Acid resistance of the wild-type strain (WT) and the Δ*mdtEF* mutant harboring vector (pHSG398) or p*ydeO* were grown to mid-log phase in LB broth (pH 7.0). Cells were diluted 40-fold using LB broth (pH 2.5) and incubated for 1 h at 37 °C. Error bars represent standard errors of the mean values.

enhanced drug efflux activity of *E. coli* cells, which is caused by the increased expression of *mdtEF*.

## DISCUSSION

In this study, we performed a genome-wide search for a regulator of multi-drug resistance of *E. coli* by random shotgun cloning and discovered YdeO, which up-regulates *mdtEF* expression, thereby increasing the resistance to β-lactams, erythromycin, rhodamine 6G and SDS. We initially found that the plasmid carrying *ydeO* conferred oxacillin resistance on the Δ*acrB* strain. Then, we investigated the susceptibility of the *ydeO*-overexpressing Δ*acrB* strain to various drugs, including the common substrates of multi-drug efflux pumps, and found that YdeO stimulates *E. coli* resistance to oxacillin,
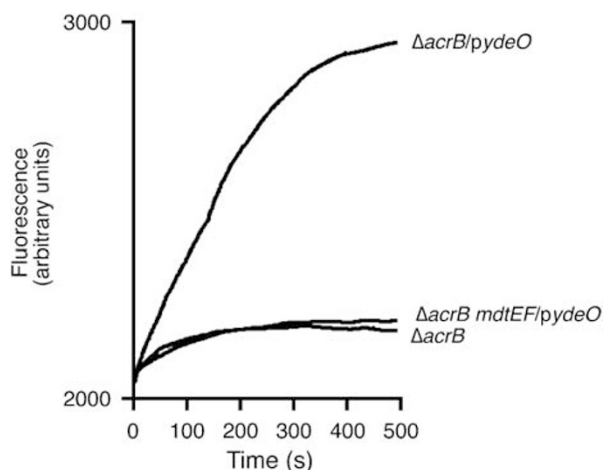
**Figure 3** YdeO enhances MdtEF-dependent efflux activity in *E. coli*. Active efflux of rhodamine 6G from *E. coli* Δ*acrB* and Δ*acrB mdtEF* cells overproducing YdeO was measured as described in the Materials and methods section.
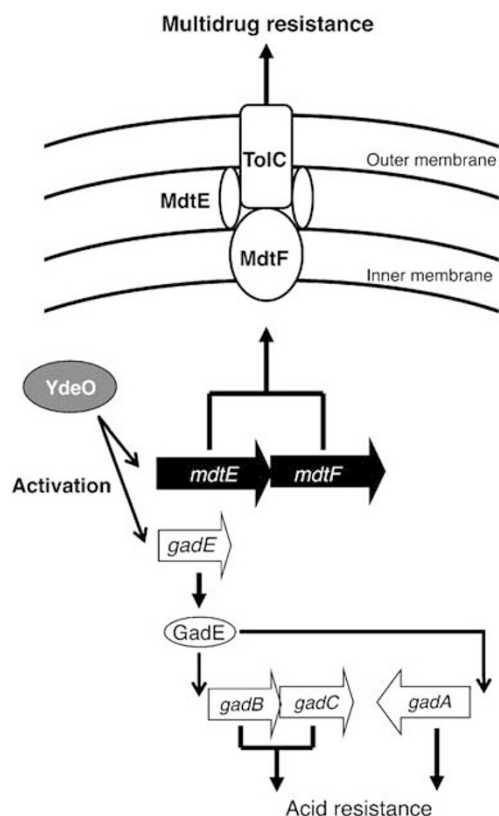


**Figure 4** Model for YdeO control of multi-drug resistance and acid resistance. YdeO controls expression of *gadABCE* acid resistance genes and *mdtEF* multi-drug efflux pump genes. The results of this study show that overexpression of *ydeO* activates expression of *mdtEF* and confers multi-drug resistance on *E. coli*.

cloxacillin, nafcillin, erythromycin, rhodamine 6G and SDS (Table 3). We discovered the importance of YdeO as a drug resistance factor through induction of the multi-drug efflux gene. Earlier, we reported that the MdtEF multi-drug efflux pump requires TolC to function.[12] YdeO-mediated multi-drug resistance was completely suppressed by deleting the *tolC* or *mdtEF* gene (Table 3). Although nothing is known of the potential YdeO-binding consensus sequence, it was reported that YdeO has an ability to bind the intergenic region between *hdeD* and *gadE*, located upstream of *mdtEF*.[36] The *gadE* and *mdtEF* genes are located in tandem and they might be co-transcribed. These facts suggest that the up-regulation of *mdtEF* expression might have occurred by the YdeO-binding to the upstream region of *gadE*.

An important feature of *E. coli* pathogenesis is the organism's ability to withstand extremely acidic environments (pH 2 or lower). This acid resistance contributes to the low infectious dose of pathogenic *E. coli* species. One very efficient *E. coli* acid resistance system encompasses two isoforms of glutamate decarboxylase (*gadA* and *gadB*) and a putative glutamate/GABA antiporter (*gadC*).[34,35] It is subject to complex controls that vary with growth medium, growth phase and growth pH. Earlier work has revealed that this system is also controlled by YdeO (Figure 4).[36] The YdeO protein is involved in transcriptional activation of the *gadE* gene coding for a protein that regulates *gadABC* (Figure 4).[36,46] YdeO belongs to the AraC–XylS family of bacterial transcriptional regulators known to activate acid resistance[36,47,48] and repress virulence.[49] We investigated the contribution of the MdtEF multi-drug efflux pump in YdeO-induced acid resistance, but MdtEF is not essential for acid resistance of *E. coli*.

In addition to the roles of YdeO in acid resistance, we found that it contributes to the multi-drug resistance of *E. coli* by activating the MdtEF multi-drug efflux pump (Figure 4). The present evidence suggests that it may enhance the resistance of *E. coli* to low pH and multiple drugs in hostile environments. Further investigation of the regulation of multi-drug efflux systems in several natural environments, such as inside hosts, is required to elucidate the biological significance of their regulatory networks. Such investigations may provide further insights into the role of multi-drug efflux systems in the physiology of the cell.

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