Growth phase-dependent transcription of *emrKY*, a homolog of multidrug efflux *emrAB* genes of *Escherichia coli*, is induced by tetracycline

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The genes *emrK* and *emrY* were found between genes *dsdA* and *evgA* at 51 min on the *Escherichia coli* chromosome and form an operon. EmrK and EmrY are 50.4 and 63.3% identical in amino acid sequences to EmrA and EmrB, respectively, which together make up a multidrug resistant pump. To show that the *emrKY* operon can be expressed, we cloned the promoter with pMC1403 and constructed an *emrK-lacZ'* protein fusion plasmid, pMKD1. In *E. coli* MC4100 containing pMKD1, its expression was increased in the presence of a subinhibitory concentration of tetracycline, chloramphenicol or salicylate, but not by carbonylcyanide *m*-chlorophenylhydrazine, nalidixic acid or kanamycin. Furthermore, we have shown that *emrKY* transcription dependent on the growth phase is actually induced by tetracycline using a S1 nuclease protection assay.

Key Words—efflux pump; *Escherichia coli*; multidrug resistance; tetracycline

Drug-resistant strains of bacteria are an increasing threat to human health. The mechanism of drug resistance involves enzymatic degradation and other modification of drugs, alteration of the drug target, or the transport of certain classes of drugs out of the cell (Davies, 1994). Some bacteria have pumps that can actively transport various compounds; the pumps are called multidrug-resistant (MDR) pumps. MDR pumps are found in a variety of bacterial species, and their presence in pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* poses a threat to public health. Their presence in *Escherichia coli* and other (generally) nonpathogenic bacteria contributes to intrinsic (background) resistance to antimicrobials (George, 1996; Lewis, 1994; Nikaido, 1996; Paulsen et al., 1996).

Many genes coding for MDR pumps have been mapped on *E. coli* chromosomal DNA (Paulsen et al., 1996), but except for *acrA*, *acrB*, *emrA*, and *emrB*, their gene expressions are not known in detail.

Recently we newly found *emrK* and *emrY* genes (Utsumi et al., 1996) which resembled *emrA* and *emrB*

(Lomovskaya and Lewis, 1992), respectively, but we did not know if they are actually expressed. In this study, we show that their expression, dependent on the growth phase, is induced by tetracycline.

Materials and Methods

Bacterial strains, plasmids and growth media. *E. coli* strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were grown at 37°C in L broth containing 10 g of polypeptone, 5 g of yeast extract, and 5 g of NaCl per liter.

Nucleotide sequencing and analysis. 16B9 DNA (a Kohara library), prepared as previously described (Kohara et al., 1987), was digested with *Pst*I. The 12-kb fragment that contained the genes *evgA* and *evgS* was extracted from the gel with a Prep-A-Gene DNA purification system (Bio-Rad Laboratories, Inc., Hercules), cloned into the *Pst*I site of pUC19 and named pKT06. We analyzed 3.2-kb of the upstream region of *evgA* using a DNA autosequencer (ABI Co., Foster City, model 373A) with Dye Deoxy Terminator cycle sequencing. The open reading frames (ORFs) were found with software from Genetyx Mac (SDC Co., Ltd., Tokyo, Japan). A search for similarity was done in the databases of GenBank and SwissPROT by

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Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
MC4100	F ⁻ <i>araD139</i> Δ (<i>argF-lac</i>) <i>U169rpsL150relA1fib5301deoC1ptsF25rbsR</i>	Shihavy et al., 1984
GC4468	F ⁻ <i>lacU169rpsL</i>	Greenberg et al., 1991
JHC1069	GC1069 <i>marR</i> (<i>cfxB1</i>) <i>zdd2207::Tn10km'</i>	Greenberg et al., 1991
JHC1096	GC4468 Δ (<i>marRAB</i>) <i>1738zee-239::Tn9cm'</i>	Greenberg et al., 1991
Plasmids		
pSK001	5.6-kb <i>HindIII</i> fragment containing <i>evgA</i> and <i>evgS</i> genes; N-terminal portion of <i>emrK</i> was cloned into pUC19	Utsumi et al., 1994
pKT06	12-kb <i>PstI</i> fragment containing <i>emrK</i> , <i>emrY</i> , <i>evgA</i> , and <i>evgS</i> genes, cloned into pUC19	This study
pKT03	<i>Fkbl</i> fragment (5.1-kb) containing <i>evgA</i> and <i>evgS</i> was removed from pKT06 and ligated by itself	This study
pMC1403	For constructing protein fusion to <i>lacZ'</i>	Casadaban et al., 1980
pMKD1	pMC1403 derivative, <i>emrK-lacZ'</i> protein fusion, Ap ^r	This study

Blastmail.

Construction of the fusion plasmid. A 523-bp fragment of pSK001 (Utsumi et al., 1994) containing the predicted promoter region and the N-terminal portion (11 amino acid residues) of *EmrK* was amplified by PCR with primer A, 5'-TAGAATTCTCCCTTTGCAATGAAGC-3' and primer B, 5'-CGGGATCCGTACTAATCATGTCTTC-3' (Fig. 1). After this amplified fragment was digested with both *EcoRI* and *BamHI*, it was ligated in frame to *lacZ'* of pMC1403 treated with both *EcoRI* and *BamHI* and used to transform *E. coli* MC4100 cells, which were selected on MacConkey plates containing lactose and ampicillin. The *emrK-lacZ'* protein fusion plasmid was named pMKD1.

β -Galactosidase assay. Stationary-phase cultures were diluted 1:100 and used to subculture fresh LB broth with the appropriate antibiotic(s). Culturing was done with shaking at 37°C. Samples were taken at the times specified hereafter for the different experiments, and the β -galactosidase activity was assayed in duplicate as described by Miller (1972).

Transcriptional analysis. Cellular RNA was extracted with sodium dodecyl sulfate-phenol at 60°C. The DNA fragment ³²P-labeled at its 5' end was hybridized to the cellular RNA, treated with S1 nuclease and analyzed as described elsewhere (Aiba et al., 1981). The probes used were prepared as follows: 478-bp containing *emrKY* promoter region was prepared from pSK001 DNA cut with *StuI* and *HpaI*. This DNA fragment was labeled with ³²P at its 5' end according to a procedure described previously (Aiba et al., 1981). After the labeled fragment was treated with *EcoRV*, the 445-bp fragment was used as the probe for the S1 nuclease protection assay shown in Fig. 4.

Primer C (5'-CCGGGATCCGTACTAATCATGTCTTC-3'), primer D (5'-TAGAATTCTCCCTTTGCAATGAA-GC-3') and pSK001 were used for PCR. PCR amplification was performed in a Perkin Elmer DNA Thermal

Cycler and used an initial step (95°C for 5 min, 50°C for 5 min, 75°C for 5 min), followed by 29 cycles at 95°C for 1 min, 50°C for 1 min, and 75°C for 3 min. The PCR product was cut with *BamHI* and *EcoRI*. This DNA fragment was labeled with ³²P at its 5' end according to a procedure described previously (Aiba et al., 1981). This labeled fragment was used as the probe for the S1 nuclease protection assay shown in Fig. 5.

Results and Discussion

Sequences of *emrKY*

Sequences 3.2-kb long on both strands containing the *emrK* and *emrY* of pKT06 were determined. Two open reading frames were found: ORF1, with 351 amino acids (MW 38,362) and ORF2 with 512 amino acids (MW 56,000). ORF1 and ORF2 had optimum scores of 949 and 1094, and were 50.45 and 63.3% identical to the multidrug efflux pump components *EmrA* and *EmrB*, respectively. They were between the *dsdA* and *evgA* genes at 51 min on the *E. coli* chromosome and constituted an operon. ORF1 and ORF2 were named *emrK* and *emrY*, respectively, and deposited as Accession No. D78108 (Utsumi et al., 1996).

Effects of drugs on *emrK-lacZ'* expression

To find if *emrKY* can be expressed, we cloned their promoter with pMC1403 to construct *emrK-lacZ'* protein fusion plasmid, pMKD1 (Fig. 1). In *E. coli* MC4100 containing pMKD1, its expression was investigated. It was increased after 24 h incubation in the presence of a subinhibitory concentration of tetracycline, chloramphenicol or salicylate, while carbonylcyanide *m*-chlorophenylhydrazine (CCCP), nalidixic acid, kanamycin and ethanol (4%) had no effect (Fig. 2).

emrAB was expressed in the presence of CCCP

Organization at 51 min region of the chromosomal DNA

Fig. 1. Construction of pMKD1.
The nucleotide sequences of primer A and primer B are described in the text.

and nalidixic acid (Lomovskaya et al., 1995). The transcription of *acrAB* was increased under general stress conditions (i.e., 4% ethanol and the stationary phase) (Ma et al., 1995). In this study, we showed that *emrK-lacZ'* expression was increased after long incubation in the presence of tetracycline, chloramphenicol or salicylate.

These results also suggest that tetracycline is the most effective drug to induce the *emrKY* operon and the tetracycline-dependent promoter of *emrKY* is located between the *emrKY* and *evgAS* operons.

Growth phase-dependent induction of *emrKY* by tetracycline

emrK-lacZ' expression was investigated for 24 h in the presence or absence of tetracycline (2 μ M). It was obviously induced at 24 h in the presence of a subinhibitory concentration of tetracycline (Fig. 3).

Furthermore, we have shown, using S1 nuclease protection assays, that the *emrKY* transcript was actually induced at 16 h when *E. coli* MC4100 containing pMKD1 was cultured in the presence of a subinhibitory concentration (2 μ M) of tetracycline (Fig. 4A). To investigate the effect of a subinhibitory concentra-

Fig. 2. Effects of drugs on *emrK-lacZ'* expression.

E. coli MC4100 containing pMKD1 was cultured for 24 h in the presence or absence of drugs as described in MATERIALS AND METHODS, and then β-galactosidase activity was assayed. Tc, tetracycline; Cm, chloramphenicol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Km, kanamycin.

Fig. 3. Growth phase-specific expression of *emrK-lacZ'*.

E. coli MC4100 containing pMKD1 was cultured for 24 h in the presence (■) or absence (▨) of tetracycline (2 μM) as described in Fig. 2, then β-galactosidase activity was assayed at the indicated times.

tion of tetracycline on cell growth, we continued to measure OD₆₀₀ for 48 h. After *E. coli* cell growth increased gradually for the first 9 h, it stopped for 10 h and resumed to increase until 35 h (Fig. 4B). These results indicate that growth-phase specific *emrKY* induction is involved in the adaptive mechanism resistant to tetracycline.

Induction after a long incubation in the presence of a subinhibitory concentration of tetracycline was specifically observed, but not in the presence of kanamycin or chloramphenicol (Fig. 5). This experiment showed that the increased expression of *emrK-*

lacZ' by chloramphenicol, as shown in Fig. 2, was due to a mechanism which is different from that induced by tetracycline.

Multiple-antibiotic-resistant (Mar) mutants of *E. coli* express elevated levels of resistance to a wide range of structurally unrelated antibiotics (George and Levy, 1983; Hächler et al., 1991). The alleles that affect the Mar phenotype are located in the *marRAB* operon (Cohen et al., 1993). The *marA* gene encodes a positive regulator of antibiotic resistance whose sequence shows strong similarity to those of such transcriptional regulators as SoxS, XylS and AraC, while *marR* en-

Fig. 4. A: Transcriptional regulation of *emrKY*.

MC4100 containing pMKD1 was cultured overnight. After 5 ml of the overnight culture was added to 500 ml of L broth in the presence of tetracycline (2 μ M) and cultured at 37°C, a 20 ml culture was sampled at 10 h (lane 1), 12 h (lane 2), 14 h (lane 3), 16 h (lane 4), 20 h (lane 5), and 22 h (lane 6), and total RNA was prepared as described previously (Aiba et al., 1981). Eighty micrograms of total RNA and the probe described in MATERIALS AND METHODS were used for S1 nuclease protection assay (Aiba et al., 1981). The arrow shows the band (198-bp) protected against S1 nuclease.

B: Effect of a subinhibitory concentration of tetracycline on growth.

After MC4100 containing pMKD1 was cultured in the presence (■) or absence (▲) of tetracycline (2 μ M) as described in MATERIALS AND METHODS, OD₆₀₀ was measured at the indicated times.

Fig. 5. Effect of drugs on the transcriptional regulation of *emrKY*.

The probe was prepared as described in MATERIALS AND METHODS. After MC4100 containing pMKD1 was cultured for 24 h at 37°C in the presence of chloramphenicol (4.6 μ M, lane 2), kanamycin (10 μ M, lane 3) or tetracycline (2 μ M, lane 4), and in the absence of drugs (lane 1), total RNA was prepared as described in MATERIALS AND METHODS. This RNA and the probe were used for S1 nuclease assay. The arrow shows the band (449-bp) protected against S1 nuclease, which corresponds to the transcript as shown in Fig. 4A.

codes a repressor of the *marRAB* operon (Cohen et al., 1993). The function of *marB* is not known.

In this study, we have investigated how the Mar system is involved in *emrKY* gene expression. In a *marR* mutant (JHC1069), *emrK-lacZ'* was increased (Fig.

6), but it was decreased in JHC1096, the mutant defective in *marRAB* (data not shown). The transcription of *acrAB* was also elevated in several *marR* mutants (Ma et al., 1995) and Okusu et al. (1996) showed that *marR1* mutation increased the resistance level, but not in the absence of the AcrAB efflux system. These results suggest that *emrKY* and *acrAB* gene expression are controlled by the Mar system.

Seoane and Levy identified four *mar* locus-regulated (*mlr*) genes, which were located at 14, 31.5, 51.6 and 77 min on the *E. coli* chromosome (Seoane and Levy, 1995). The gene at 77 min was identified as lipoprotein *slp* (Seoane and Levy, 1995). The other three have not been sufficiently characterized. From DNA sequences, these genes are obviously not identical to *emrKY*.

Martin et al. showed that salicylate appears to induce the *mar* operon by binding to MarR and inhibiting complex formation, while tetracycline and chloramphenicol, which neither bind MarR nor inhibit complex formation, must induce by an indirect mechanism (Martin and Rosner, 1995). In MC8301 (Utsumi et al., 1993) (MC4100, *rpoS::Tn10*), *emrK* expression was decreased by tetracycline, but not by chloramphenicol or salicylate (data not shown). These results suggest that *emrKY* gene expression is controlled by both RpoS and the Mar system, which is different from the mechanism for chloramphenicol and salicylate.

emrKY from pKT06 was cloned into pUC19 to con-

Fig. 6. Involvement of MarR in *emrK-lacZ'* expression.

E. coli GC4468 (*marR*⁺) (□) and JHC1069 (*marR*) (■) containing pMKD1 were cultured to 0.4 OD₆₀₀ and for 24 h in the presence or absence of tetracycline (1 µg/ml) as described in MATERIALS AND METHODS, and then β-galactosidase activity was assayed.

struct pKT03 (Table 1). HN971 (*acrA1* and *emrB::kan*) (listed as HN791, Thanassi et al., 1997) containing pKT03 or pUC19 was incubated for 24 h in the presence of tetracycline to measure their minimal inhibitory concentrations (MIC). The MICs of HN971 containing pKT03 and pUC19 were 2 µM (1 µg/ml) and 0.5 µM (0.25 µg/ml), respectively. These results suggest that EmrKY worked as the efflux pump of tetracycline.

The cloned *emrY* gene belongs to a superfamily of membrane transporters, the major facilitator superfamily (MFS). Although many members of the MFS are involved in solute import, we believe that *emrY* is involved in the efflux of solute(s) because of two reasons: (a) EmrY is predicted to contain 14 transmembrane segments (TMS), and all of the known 14 TMS members of MFS are exporters of substrates (Paulsen et al., 1996); and (b) EmrY contains some signature sequences found only in drug/proton antiporter members of the MFS, such as the G-X-hy-hy-G-P-X-I-G-G (where hy indicates a hydrophobic residue) (Paulsen et al., 1996), which starts from ala154 in EmrY (A-P-I-C-G-P-I-L-G-G). These observations do not necessarily indicate that EmrY is an MDR transporter, because these two features are also found in single-drug efflux proteins of the MFS family (Paulsen et al., 1996). However, the sequence of EmrY is more closely related to a known MDR transporter, EmrB, than to any other single-drug efflux protein (Paulsen et al., 1996), and this seems to suggest that EmrY also functions as a multidrug efflux transporter. The identity of the real substrates of the EmrKY system, however, is not yet

clear.

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