Note

Resistance to Cationic Surfactants and Some Other Agents of *Escherichia coli* Recombinants with an Abundant Number of Genes Encoding Drug Efflux Pump

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In order to investigate the contribution of the multidrug efflux pumps to cellular resistance to a variety of agents including cationic surfactants, we cloned genes of major drug efflux pumps of *Escherichia coli* and transformed strain JM109 with plasmids encoding those genes. In *emrAB*-, *mdfA*- and *emrE*-recombinants, the resistance to carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), chloramphenicol, and paraquat, respectively, was increased compared to their parent bearing pUC18. Also, in *mdfA*- and *emrE*-recombinants, the resistance to cetyltrimethylammonium bromide (CTAB) was increased. However, no significant effects of abundant numbers of *emrD* and *envCD* could be observed on resistance to agents examined.

Key words : Multidrug efflux pump/Cationic surfactant resistance/*E. coli*.

Surfactants as amphipathic compounds have been utilized in a variety of fields, such as chemical, pharmaceutical and food industries, hospitals, homes, and environments for cleaning, emulsification, solubilization, moisturizing and so on (Schwartz and Perry, 1979). In particular, cationic surfactants have antimicrobial activity (Hugo and Russell, 1992; Schwartz and Perry, 1979) and are therefore widely used in hospitals and food industries as disinfectants or sanitizers. As for the mode of action, they have been reported to disrupt cell membranes (Scharff and Maupin, 1960), interrupt protein functions (Putnam, 1948), release intracellular K⁺ and other constituents (Cabral, 1993; Sakagami et al., 1989), inhibit cellular respiration (Majtan et al., 1995) and induce cell autolysis (Cho et al., 1990; Tsuchido et al., 1990). However, several investigators have isolated so far cationic surfactant resistant-bacteria from a variety of niches (Heir et al., 1995, 1998 and 1999; Kücken et al., 2000; Lemaitre et al., 1998; Littlejohn et al., 1992;

Nagai et al., 1996; Sakagami et al., 1989).

Major ways that bacterial cells acquire drug resistance are as follows: i) inactivation of the drug, ii) characteristic or quantitative change of the target of the drug, iii) depression of drug permeation and iv) active drug efflux (Davies, 1994). Among these ways, drug efflux from cells has recently been actively investigated and many membrane translocators have been found as efflux pumps. Their function is known to confer multidrug resistance to bacterial cells (Lewis, 1994; Nikaido, 1994 and 1996). Such drug efflux pumps can extrude a variety of agents, including antibiotics, surfactants, oxidative phosphorylation uncouplers, and oxidants from cells. Lacroix et al. (1996 and 1995) have found that an *acrB*-like gene confers not only multiple antibiotic resistance but also multiple surfactant resistance on *Salmonella typhimurium* cells. Several investigators have also reported that overproduction of drug efflux pumps increases cellular resistance to various agents including surfactants (Edgar and Bibi, 1997; Jack et al., 2000; Neyfakh et al., 1991). We have also isolated and characterized a cationic surfactant resistant strain of

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Escherichia coli and found that the strain has an altered cell envelope compared to its parent strain (Ishikawa et al., 2002).

In this study, to investigate a possible contribution of increased numbers of efflux pump genes to the cellular resistance to a variety of agents including cationic surfactants, we cloned genes of major drug efflux pumps referred to in GenBank, *emrAB* (AE000152), *emrD* (AE000445), *mdfA* (*cmr*; AE000186), *emrE* (*mvrC*; AE000160) and *envCD* (*acrEF*; AE000405), from the chromosomal DNA of *E. coli*, and then examined how much efflux pump gene-recombinants of *E. coli* increase their resistance to cationic surfactants and some other agents.

Genes of drug efflux pumps of *E. coli* including their original promoters and terminators amplified by the PCR method were cloned in multi-cloning sites of pUC18/19 or pHSG575 (Table 1). Chromosomal DNA of *E. coli* OW6 (Pro⁻) (Kitagawa et al., 2000) as

a template and primer sets designed from the *E. coli* genome sequence were employed (Table 2). Strain JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ (*lac-proAB*)/F' [*traD36*, *pro*⁺, *lacI*^q, *lacZ* Δ M 15]) (Sambrook et al., 1989) was used as a host strain. Recombinants were cultivated in LB medium (pH 7.4), consisting of 10 g bacto tryptone (Difco Laboratories, Detroit, USA), 5 g yeast extract (Difco Laboratories), and 5 g NaCl per liter, at 37°C with shaking at 120 rpm with or without an antimicrobial agent added. The minimum growth inhibitory concentration (MIC) of agents was determined by cultivation using LB medium with shaking at 37°C for 18 h as previously described (Ishikawa et al., 2002). Membrane proteins were extracted by sonication following the method of Fukuoka et al. (1991), and analyzed by SDS-PAGE (Laemmli, 1970). The proportion of plasmid retention in the host strain was determined as follows. After a portion of the overnight culture was di-

TABLE 1. Recombinant plasmids employed in this study.

Plasmid	Description	Substrates of cloned drug efflux pump ^a	Source or Reference
pUC18/19	High-copy-number vectors, Ap ^r		Yanisch-Perron et al., 1985
pHSG575	Low-copy-number vector, Cm ^r		Takeshita et al., 1987
pEAB1906	pUC19 carrying <i>emrAB</i>	CCCP, phenylmercury acetate, NAX	This study
pED1906	pUC19 carrying <i>emrD</i>	CCCP, phenylmercury acetate	This study
pMA1806	pUC18 carrying <i>mdfA</i>	Cm, Tc, Em, BC, EB, and others	This study
pEE1806	pUC18 carrying <i>emrE</i>	Lipophilic cations (PQ, EB, TPP)	This study
pECD4806	pHSG575 carrying <i>envCD</i>	Em, Tc, Ap, SDS, CV, and others	This study

^a Substrates were referred to in reports by Nikaido (1994 and 1996). Abbreviations: NAX, Nalidixic acid; Cm, chloramphenicol; Tc, tetracycline; Em, erythromycin; BC, benzalkonium chloride; CV, crystal violet; EB, ethidium bromide; PQ, paraquat; TPP, tetraphenylphosphonium; Ap, ampicillin; SDS, sodium dodecyl sulfate.

TABLE 2. Oligonucleotide primers used to amplify MDR genes.

Gene	Name ^a	Sequence of oligonucleotide (5' to 3') ^b
<i>emrAB</i>	emrAB-U	CGTCTTTG <u>CGATGC</u> ACATGCA (<i>Sph</i> I)
	emrAB-D	GAAAGAGGGATCCATACCCCT (<i>Bam</i> H I)
<i>emrD</i>	emrD-U	TGCACAAaAGCTtGCAACAG (<i>Hind</i> III)
	emrD-D	CCCAGGACTGCAGCCGGAGC (<i>Pst</i> I)
<i>mdfA</i>	mdfA-U	AAAAATCGtCgACGAGATCA (<i>Sal</i> I)
	mdfA-D	CGAACACaAGcTTGACGACC (<i>Hind</i> III)
<i>emrE</i>	emrE-U	CGATGCCCTGCAGATGGCGC (<i>Pst</i> I)
	emrE-D	CTGATTGTGCATGCTTTCCT (<i>Sph</i> I)
<i>envCD</i>	envCD-U	ATGTCGTTGAGCGTCGaCTTGCTTACGCCA (<i>Sal</i> I)
	envCD-D	ATTGTAAAAATaTGcAGGGAGCGGGAACCT (<i>Pst</i> I)

^a U and D indicate primers for the upstream region and downstream region, respectively.

^b Restriction sites were indicated as underlined letters, and small letters indicate mutation introduced.

luted, cells were spread on the LB agar plate with or without the marker antibiotic. Then, plates were incubated at 37°C for 16 h. The index of plasmid stability was calculated as a ratio of colony forming units on the antibiotic-containing plate versus that on the plain LB plate.

Recombinants grew in the plain LB medium as did the parental strain bearing pUC18. Percentages of plasmid retention in all recombinants were over 80%, even if recombinants were cultivated in the absence of a plasmid selective marker. This result indicates that each plasmid is highly stable in the host cell, and thereafter recombinants were cultivated without addi-

tion of a plasmid marker when their drug resistance was evaluated.

We examined the overproduction of the products of *emrAB* genes in JM109/pEAB1906 after cultivation in CCCP (50 μ M)-containing medium. Though an increase in the signal of EmrB was not found at the position corresponding to a molecular mass of 56 kDa, overproduction of EmrA was detected (Fig. 1).

Resistance of efflux pump gene-recombinants to agents as efflux substrates listed in Table 1 was examined by using both the MIC test and the descriptive turbidity monitoring method. The MIC of CCCP for the *emrAB*-recombinant was twice and that of chloramphenicol for the *mdfA*-recombinant was 7 times as much as that of the parental strain bearing pUC18 (Table 3). In the *emrD*-recombinant, the MIC of CCCP was scarcely increased (Table 3). Further, both the *emrAB*- and *mdfA*-recombinants grew in a medium containing CCCP (100 μ M) and chloramphenicol (20 μ g/ml), respectively, without any delay in growth, whereas their parental strain could not grow under the same conditions (Fig. 2A and B). Although increased resistance to paraquat was not found in the *emrE*-recombinant when the MIC method was used, a difference in the growth pattern was observed when the turbidity was monitored during the early stages of cultivation. The *emrE*-recombinant grew in the medium containing paraquat at a final concentration of 50 μ M, whereas the growth of its parental strain was inhibited markedly (Fig. 2C). However, in the *envCD*-recombinant, we failed to find increased resistance to ampicillin, tetracycline, erythromycin and crystal violet (Table 3 and data not shown), which have been known as efflux substrates (Nikaido, 1994).

To investigate the surfactant resistance of efflux pump gene-recombinants the following agents were

FIG. 1. SDS-PAGE analysis of membrane proteins. Membrane fraction extracted from an overnight culture of strain JM109/pEAB1906 grown in LB medium containing CCCP at a final concentration of 50 μ M. Lane 1: membrane fraction of the parental strain grown in plain LB medium, Lane 2: membrane fraction of JM109/pEAB1906 grown in LB medium containing CCCP. The arrow indicates a signal of EmrA.

TABLE 3. MICs^a of various agents against *E. coli* transformants carrying various drug efflux pump genes.

Plasmid	Gene carried	Ap (μ g/ml)	Cm (μ g/ml)	Tc (μ g/ml)	CV (μ g/ml)	CCCP (μ M)	PQ (μ M)	CTAB (μ M)	BC (μ M)	CPC (μ M)
pUC18	—	ND ^b	10	4	>10	150	>500	30	20	20
pEAB1906	<i>emrAB</i>	ND	10	4	>10	300	>500	20	20	10
pED1906	<i>emrD</i>	ND	15	6	>10	200	>500	20	20	20
pMA1806	<i>mdfA</i>	ND	70	6	>10	150	>500	20	20	20
pEE1806	<i>emrE</i>	ND	10	4	>10	100	>500	40	30	20
pHSG575	—	18	ND	6	>10	150	>500	20	20	10
pECD4806	<i>envCD</i>	6	ND	6	>10	150	>500	20	20	20

^a Bold letters indicate substrate(s) of each efflux pump, and names of drugs are abbreviated as follows; Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline; CV, crystal violet; PQ, paraquat; BC, benzalkonium chloride; CPC, cetylpyridinium chloride.

^b Not done; the agent corresponds to plasmid marker.

FIG. 2. Growth of *E. coli* JM109 carrying plasmids encoding different efflux pump genes in the presence of 100 μ M CCCP (A), 20 μ g/ml chloramphenicol (B), 50 μ M paraquat (C) and 15 μ M CTAB (D). Strain JM109 harboring a plasmid pUC18 ($\circ$), pEAB1906 ($\triangle$, $\blacktriangle$), pMA1806 ($\square$, $\blacksquare$), or pEE1806 ($\diamond$, $\blacklozenge$), was cultivated in L medium at 37°C. Closed and open symbols indicate growth in the medium with and without addition of the drug, respectively.

employed: CTAB (Wako Pure Chemical Industries, Ltd., Osaka), benzalkonium chloride (Dojindo Laboratories, Kumamoto), cetylpyridinium chloride (Wako), benzethonium chloride (Wako) as cationic types, and sodium dodecyl sulfate (SDS, Wako) and sodium deoxycholate and 3[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfate (CHAPS, Dojindo Laboratories) as anionic and zwitterionic types. For all surfactants examined, no significant differences in the MIC were observed between each recombinant and its parental strain (Table 3 and data not shown). In the turbidity monitoring, however, the *mdfA*- and *emrE*-recombinants grew after a short lag even in the presence of 15 μ M CTAB, whereas the parental strain did not (Fig. 2D).

It is generally considered that the activity of the drug efflux pump directly corresponds to the degree of cellular drug resistance (Nikaido, 1994 and 1996). In fact, in this study, the *mdfA*- and *emrE*-recombinants were demonstrated to have multidrug resistance. However, no marked multidrug resistance was observed in each recombinant tested here. One possible reason may be due to the nature of the host strain JM109 itself. This strain has a full set of genes of drug efflux pumps, including genes used in this study, on its chromosome. We infer that the drug resistance of strain JM109 is relatively high and this high basal resistance may mask an increase in drug resistance mediated by each amplified gene of pump. In fact, when an *acrAB* mutant of *E. coli*, which has low basal resistance, is used to clone efflux pump genes, markedly increased resistance has been obtained (Masaoka et al., 2000; Mine et al., 1999; Morita et al., 1998).

Very recently, characterization of knock-out mutants (Sulavik et al., 2001) and overproducing strains (Nishino and Yamaguchi, 2001) of various drug efflux genes in *E. coli* have been investigated. However, it remains unclear whether and how those genes are involved in the appearance of drug resistant bacteria like those isolated from hospitals or food industries.

We have isolated a CTAB-resistant mutant from *E. coli* and reported that the mutant was multidrug resistant (Ishikawa et al., 2002). We cloned the same sets of drug efflux pumps described here from the mutant and investigated the degree of increased resistance of recombinants having those genes. However, the effect of gene dosage efflux pump genes derived from this mutant were the same level as those derived from the parent strain OW6 (data not shown). In this mutant, therefore, it is suggested that some mechanism other than drug efflux may also work for its multidrug resistance.

Recently, several mutants resistant to sanitizers including cationic surfactants have been isolated and some of them were characterized to possess multiple antibiotic resistant property (Akimitsu et al., 1999; Ishikawa et al., 2002; Tattawasart et al., 1999). This finding predicts a possible risk upon resulting from continuous use of surfactants, that is, the occurrence of multidrug resistant mutants from bacteria inhabiting various niches. Moreover, Miyamae et al. (1998) have reported a norfloxacin-resistant mutant of *Bacteroides fragilis* isolated spontaneously which increased its resistances not only to several antibiotics but also to CTAB. The cross resistance between sanitizers including surfactants and antibiotics does not seem to be a peculiar phenomenon, since

bacteria easily acquire multidrug resistance, as exemplified previously (Ishikawa et al., 2002). In the near future, these mutants might become a serious environmental problem in human life. In order to understand the detailed mechanisms of bacterial multidrug resistance, further studies are necessary.

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