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Characterization and Protein Analysis of *Escherichia coli* Resistant to a Quaternary Ammonium Compound

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A resistant strain of *Escherichia coli* IFO12713, resistant against the quaternary ammonium compound (QAC), *N*-dodecylpyridinium iodide (P-12), was developed by using a standard broth dilution method and physiologically characterized by comparison to a sensitive strain. The cell surface hydrophobicity cultivated in the resistance-acquiring process gradually decreased and the lipopolysaccharides in the outer membrane increased. This result indicated that the cell surface of the resistant strain was more hydrophobic than that of the sensitive strain. One of the resistant strains isolated from the process also had resistance to other antibiotics and organic solvents. Rapid ethidium efflux was observed in the resistant strain compared to the sensitive strain. To elucidate the mechanism of resistance on a molecular level, protein analysis was performed with 2D-PAGE for the entire cellular fraction, and SDS-PAGE for the outer or inner membrane fractions. It was seen that 18 spots and bands of proteins specifically increased in the resistant strain. From the physiological characterization and the protein analysis, it was suggested that the QAC-resistant strain acquired its resistance by responding to antimicrobial stresses, stabilizing the outer membrane to control the adsorption/binding reaction between the cell and agent, and activating the efflux pump against the antimicrobial agents.

Key words : Quaternary ammonium compound/*Escherichia coli*/Biocide resistance/Cell surface hydrophobicity/Efflux pump/Stress response.

INTRODUCTION

In the modern world, we have various techniques of microbial control to solve the problems caused by harmful microbes which contribute to food poisoning, hospital infections, microbial deterioration and so on. Among these techniques, the development and use of antimicrobial agents is one of the most important. Since there are numerous kinds of antimicrobial agents, the use of a suitable agent in the right way based on its antimicrobial potentiality or chemical and physical characteristics is necessary. Quaternary ammonium compounds (QACs), cationic surfactants,

have been widely utilized for medical, industrial and domestic use because of their low toxicity or wide antimicrobial spectrum (Baley et al., 1977; Merianos, 2001). However, their utilization has brought about environmental pollution, harmful effects on humans and the growth of resistant bacteria. Therefore, it is necessary to elucidate the resistance mechanism of bacteria and development of novel biocides which do not produce resistant bacteria.

There are many reports about bacteria resistant to QACs, i. e., *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus* spp., etc. (Aase et al., 2000; Alekshun and Levy, 2000; Heir et al., 1995; Ishikawa et al., 2002a and 2002b; Sakagami et al., 1989; Tabata et al., 2002, 2003a and 2003b). Generally, it is important to take measures against the emergence

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of Gram-negative bacteria, which are resistant to biocides or antiseptics in nature due to their cell surface structure, compared with Gram-positive bacteria (Denyer and Maillard, 2002). Major mechanisms of bacterial resistance to antimicrobial agents are known as 1) impermeability, 2) efflux, 3) modification of the target site, and 4) drug inactivation (Poole, 2002). Among them, possible ways to resist biocides involve impermeability and efflux (Russell, 2001). It is known that impermeability is due to the cell surface structure, particularly, the lipopolysaccharides of Gram-negative bacteria (Sukupolvi and Vaara, 1989). Drug efflux systems which consist of membrane proteins are called efflux pumps.

In this paper, we studied the characteristics of an *E. coli* strain resistant to a QAC, analyzed the cellular proteins, and investigated the mechanism of resistance.

MATERIALS AND METHOD

Chemicals

As a typical mono-QAC, *N*-dodecylpyridinium iodide (P-12) (Kourai et al., 1980) was synthesized in our laboratory. Benzalkonium chloride was obtained from Kanto Chemical Co., Inc. (Tokyo). Cetylpyridinium chloride was supplied by Tokyo Kasei Kogyo Co., Ltd. (Tokyo). TEGO-51 was purchased from Alfresa Pharma Corp. (Osaka). Methicillin, erythromycin, lincomycin, doxycycline and chloramphenicol were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Penicillin G, streptomycin, kanamycin and tetracycline were supplied by Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals were purchased from known suppliers and used without further purification.

Organism and culture conditions

The bacterial strain used for the experiment was *E. coli* IFO12713. It was cultured according to a previous report (Maeda et al., 1996).

Measurement of antimicrobial activity

The minimum inhibitory concentration (MIC) of the biocides was basically estimated according to the standard broth dilution method (Maeda et al., 1996).

Development of *E. coli* with adapted resistance to P-12

The adaptation of *E. coli* to QAC was basically carried out according to a previous report (Tabata et al., 2002).

Estimation of organic solvent resistance

The organic solvent resistance of *E. coli* was estimated according to a previous report (Aono and Kobayashi, 1997). Stationary phase cells (10^7 cells) were plated on LBGMg agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.1% glucose, 10mM MgSO₄, 1.5% agar, pH 7.0) overlaid with *n*-hexane ($\epsilon = 1.89$), cyclohexane ($\epsilon = 2.05$), toluene ($\epsilon = 2.24$) and *p*-xylene ($\epsilon = 2.27$). The parameter, ϵ , is the permittivity of the solvent (Asahara et al., 1976). The permittivity represents the affinity to the polar liquid, i. e., hydrophobicity. After incubation for 24 h at 37°C, the growth of *E. coli* was estimated by visual inspection.

Measurement of cell surface hydrophobicity

The measurement of cell surface hydrophobicity and calculation of the hydrophobicity index (log HI) were basically performed according to a previous report (Kourai et al., 1989).

Measurement of the amount of lipopolysaccharides

The measurement of the amount of lipopolysaccharides (LPS) on the cell surface was performed according to a previous report (Kourai et al., 1994).

Measurement of efflux activity

The measurement of the efflux activity was done according to a previous report (Masaoka et al., 2000). The *E. coli* cells were harvested at the late exponential phase, washed twice with M9 minimal medium and suspended in the same medium to an optical density of 0.2 at 660 nm. Carbonylcyanide *m*-chlorophenylhydrazone (40 μ M) and ethidium bromide (2.5 μ M) were added to the cell suspension. The cell suspension was incubated for 1 h at 37°C to deplete the energy of cells and to load the cells with ethidium. Then, cells were harvested and washed twice with the minimal medium supplemented with ethidium bromide (2.5 μ M, final concentration) and resuspended in the same medium to an optical density of 0.1 at 660 nm. The cell suspension was preincubated at 37°C for 5 min, and the assay was started. The fluorescence intensity of the assay mixture was measured at the excitation and emission wavelengths of 500 and 580 nm, respectively, using the fluorescence spectrophotometer (F-3010, Hitachi Co., Ltd., Tokyo).

Analysis of proteins

The outer membrane fraction was prepared by the method of Nakajima et al., (1998). The cells of the resistant strain and the sensitive strain were cultured in nutrient broth containing P-12 (60 ppm, half of MIC)

and fresh nutrient broth, respectively. Both of them were harvested at the late exponential phase. The outer membrane protein profile was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and two-dimensional PAGE (2D-PAGE) in an electrophoresis apparatus (TEP-2, Shimadzu Co., Ltd., Kyoto). The electrophoresed gels were stained with silver nitrate. Sequencing of the amino acid sequences of the outer membrane proteins extracted from the acrylamide gel fragments from 2D-PAGE was carried out by a protein sequencer (PPSQ-10, Shimadzu Co., Ltd., Kyoto). Homology analysis of the amino acid sequences was performed with the protein database search programs, BLAST (<http://blast.genome.jp/>) and FASTA (<http://fasta.genome.jp/>). The isoelectric point of predicted proteins was calculated from the amino acid composition with a genetic information analyzing computer application (GENETYX, Software Development Co., Ltd., Tokyo).

RESULTS AND DISCUSSION

E. coli with adapted resistance to P-12

The development of the adapted resistance of *E. coli* to P-12 is shown in Fig. 1. The MIC constantly increased and reached the maximum of 120 ppm (about 4-fold higher than MIC for the wild strain) after the eighth adaptation cycle. Therefore, it was shown that *E. coli* acquired a resistance to P-12 through repetition of the adaptation cycle. Since the MIC values did not change using the adapted bacterial strain recultured in fresh medium, the adapted resistance to P-12 was proved to be stable. In the adaptation process, there are various strains of different sensitivity to

FIG. 1. Relationship between the adaptation cycle and MICs of P-12 for *E. coli*.

TABLE 1. MICs of various antimicrobial agents against the *E. coli* IFO12713 sensitive strain and resistant strain.

Antimicrobial agents	MIC (ppm)		
	Sensitive	Resistant	Fold
Benzalkonium chloride	3.9	7.8	2.0
Cetylpyridinium chloride	5.9	15	2.5
TEGO-51	7.8	16	2.1
Penicillin G	24	150	6.3
Methicillin	900	5400	6.0
Erythromycin	370	750	2.0
Lincomycin	760	2900	3.8
Streptomycin	0.47	0.58	1.2
Kanamycin	0.14	0.27	1.9
Tetracycline	1.4	2.8	2.0
Doxycycline	3.9	6.2	1.6
Chloramphenicol	4.6	7.1	1.5

P-12. Then, the strain able to grow in the presence of 120 ppm of P-12 was isolated from the culture at the tenth cycle, and the isolate was used for the experiment as the resistant strain. Specially for the analysis of cell surface hydrophobicity and LPS, the cultures of each adaptation cycle were used in order to investigate the typical characteristics of the various strains in the culture.

MICs of various antimicrobial agents

The MICs of various antimicrobial agents were measured. The resistant strain was more resistant than the sensitive strain to certain antibiotics, i. e., penicillin G, methicillin, erythromycin, lincomycin, streptomycin, kanamycin, tetracycline, doxycycline and chloramphenicol (Table 1). Those antibiotics have different sites of inhibition or modes of action. To benzalkonium chloride and cetylpyridinium chloride as cationic surfactants, and TEGO-51 as a zwitterionic surfactant, the resistant strain was more resistant than the sensitive strain. Thus it is suggested that the resistance to P-12 is not due to modification of the target sites or inactivation of the antibiotic but other mechanisms, i. e., decreased permeability or activated efflux.

Organic solvent resistance

The organic solvent resistance of the QAC-resistant strain was investigated (Fig. 2). The resistant strain was able to grow well in *n*-hexane while the sensitive strain grew little. In cyclohexane, the resistant strain grew well, but the sensitive strain did not, and both of the strains did not grow in toluene and *p*-xylene (data not shown). Therefore, the resistant strain was tolerant to relatively hydrophobic solvents, compared with the sensitive one. Since the organic solvent resistance is due to the barrier of the cell surface structure or activation of the efflux pump, the

FIG. 2. Organic solvent resistance of *E. coli* IFO12713 sensitive strain (S) and resistant strain (R). The tested organic solvent was *n*-hexane.

result mentioned above may have relevance to the antibiotic resistance in Table 1.

Cell surface hydrophobicity and lipopolysaccharide

Since the alteration of the hydrophobic interaction was important for the resistance to P-12 (Tabata et al., 2003a), the change in the cell surface hydrophobicity in the resistance-acquiring process was investigated (Fig. 3). Cell surface hydrophobicity gradually decreased in the process, and the surface hydrophobicity of the resistant strains was more hydrophilic than that of the sensitive strains. As the factor most likely affecting the cell surface hydrophobicity is thought to be the LPS (Denyer and Maillard, 2002), the amount of LPS was measured. The amount of LPS of the outer membrane increased with the decreasing hydrophobicity in the resistance-acquiring process (Fig. 4). LPS consists of a hydrophobic lipid and hydrophilic polysaccharides, and is a component of the outer membrane of gram-negative

FIG. 3. Relationship between the adaptation cycle and cell surface hydrophobicity of *E. coli*.

FIG. 4. Relationship between the adaptation cycle and the amounts of LPS of *E. coli*.

bacteria (Sukupolvi and Vaara, 1989). The hydrophilic characteristics of the resistant strain may be due to the extension of the polysaccharide chain or an increase in the amount of LPS. In any case, it seems that the change in the LPS obstructs the contact of P-12 to the bacterial surfaces.

In Fig. 1, the MIC reached the ceiling at eighth cycle, while the tendencies seen in Figs. 3 and 4 were different from it. If the mechanism of P-12 resistance is exclusively caused by the change in cell surface hydrophobicity or LPS, such a phenomenon cannot be observed. Hence it is reasonable that there are resistance mechanisms other than the alteration of outer membrane.

Efflux activity

Based on the result mentioned above, it is suggested that multidrug efflux pumps are activated in the QAC-adapted *E. coli*. Ishikawa et al. (2002a) reported that EmrE, a typical small multidrug resistance (SMR) family multidrug efflux pump, increased the resistance to cetyltrimethylammonium bromide and benzalkonium chloride, popular cationic QACs. In another report (Masaoka et al., 2000), the ethidium efflux assay was used to estimate the activity of EbrAB which is a SMR family multidrug efflux pump. Therefore, to investigate the activation of multidrug efflux pumps in the QAC-adapted *E. coli*, the ethidium efflux was measured. Energy starved cells were first loaded with ethidium, and glucose was added to energize the cells. As shown in Fig. 5, a rapid ethidium efflux was observed with the resistant cells just after the addition of glucose. On the other hand, only a slow efflux of ethidium was observed with the normal cells. This result supports the idea that one of the

FIG. 5. Assay of ethidium efflux activity. The dashed and solid lines indicate the cellular ethidium accumulation in the *E. coli* sensitive strain and resistant strain, respectively.

resistant mechanisms of the QAC-adapted *E. coli* is an acceleration of the drug efflux.

Protein analysis

To investigate the resistance mechanism on a molecular level, a protein analysis was performed with 2D-PAGE for the entire cellular fraction (Fig. 6) and SDS-PAGE for the outer and inner membrane fractions (Figs. 7A and 7B). Consequently, the amounts of 18 spots and bands of proteins specifically increased in the resistant strain.

As for the entire cellular fraction, 9 proteins were identified (Table 2). Tryptophanase and isocitrate dehydrogenase are related to the TCA cycle, and the 6-phosphogluconate dehydrogenase to pentose-

FIG. 6. 2D-PAGE analysis of the entire cellular proteins of the *E. coli* sensitive strain (S) and resistant strain (R). The amount of proteins loaded onto each gel was 100 μ g. The circles indicate the spots of proteins which specifically increased in the resistant strain. The numbers close to the circles correspond to those of Table 2.

FIG. 7. SDS-PAGE analysis of the outer membrane proteins (A) and the inner membrane proteins (B) of *E. coli* sensitive strain (S) and resistant strain (R). The amount of proteins loaded onto each well was 10 μ g. The bars indicate the bands of proteins which specifically increased in the resistant strain. The number of bars corresponds to those in Tables 3A and 3B.

phosphate cycle, which functions to produce energy. Cytidine deaminase and adenine phosphoribosyl transferase are concerned with nucleic acid metabolism, and oxygen-insensitive NAD(P)H nitroreductase with amino acid metabolism. Superoxide dismutase and peptidyl-prolyl cis-trans isomerase B are stress responding proteins. The function of the modulator of drug activity B is unknown.

As for the outer and inner membrane fractions, 4 and 8 proteins were identified (Tables 3A and 3B), respectively. The outer membrane protein TolC, maltoporin, and long chain fatty acid transport protein are porins, which play the role as an efflux pump. The function of the outer membrane protein OmpX is adhesion to mammalian cells. The aerobic glycerol-3-phosphate dehydrogenase, ATP synthase α chain and ATP synthase β chain are related to energy production. The outer membrane protein TolB stabilizes the outer membrane. The D-alanyl-D-alanine carboxypeptidase fraction A functions during the synthesis of peptidoglycan, which is a major component of the cell surface structure.

From the physiological characterization and the protein analysis, it was suggested that the QAC-resistant strain acquired its resistance by responding to antimicrobial stresses (superoxide dismutase and peptidyl-prolyl cis-trans isomerase B), stabilizing the outer membrane to control the adsorption/binding reaction between the cell and agent (OmpX and D-alanyl-D-alanine carboxypeptidase), and activating

TABLE 2. Proteins identified by amino acid sequences from 2D-PAGE separation of resistant *E. coli* entire cellular fractions.

Spot	Amino acid sequences	Name	Function	Mw (kDa)	pI
1	MENFKHLPEPFR	TnaA	Tryptophanase (EC.4.1.99.1)	52.8	5.85
2	MESKVVVPAQG	Idh	Isocitrate dehydrogenase (EC.1.1.1.42)	45.8	5.01
3	SKQQIGVVGMV	6Pg	6-Phosphogluconate dehydrogenase (EC.1.1.1.44)	51.5	4.90
4	MHPRFQTAFAL	Cdd	Cytidine deaminase (EC.3.5.4.5)	31.5	5.35
5	MDIISVALKRHS	NfnB	Oxygen-insensitive NAD(P)H nitroreductase (EC.1.6.99.7)	23.9	5.78
6	SYTLPSLPYAYD	SodM	Superoxide dismutase (EC.1.15.1.1)	23.3	6.51
7	SNILIINGAK	MdaB	Modulator of drug activity B	21.9	5.84
8	TATAQQLEYLKN	Apt	Adenine phosphoribosyl transferase (EC.2.4.2.7)	19.9	5.13
9	MVTFTHTNHGDIV	PpiB	Peptidyl-prolyl cis-trans isomerase B (EC.5.2.1.8)	18.2	5.43

Name, function and molecular weight (Mw) were obtained by homology analysis of the amino acid sequences, and the isoelectric point (pI) was calculated from the amino acid composition of the predicted proteins.

TABLE 3A. Proteins identified by amino acid sequences from SDS-PAGE separation of resistant *E. coli* outer membrane fractions.

Band	Amino acid sequences	Name	Function	Mw (kDa)	pI
1	ENLMQVYQQARL	TolC	Outer membrane protein TolC	54.0	5.34
2	ENLMQVYQQARL	TolC	Outer membrane protein TolC	54.0	5.34
3	VDFHGYARSGIG	LamB	Maltoporin precursor	49.9	4.66
4	AGFQLNEFS	FadL	Long-chain fatty acid transport protein	48.8	5.06
5	ATSTVTGGYAS	OmpX	Outer membrane protein OmpX	18.7	7.11

Name, function and molecular weight (Mw) were obtained by homology analysis of the amino acid sequences, and the isoelectric point (pI) was calculated from the amino acid composition of the predicted proteins.

TABLE 3B. Proteins identified by amino acid sequences from SDS-PAGE separation of resistant *E. coli* inner membrane fractions.

Band	Amino acid sequences	Name	Function	Mw (kDa)	pI
1	METKDLIVIGV	GlpD	Aerobic glycerol-3-phosphate dehydrogenase (EC.1.1.99.5)	57.3	7.09
2	MQLNSTEISE	AtpA	ATP synthase α chain (EC.3.6.3.14)	55.3	5.74
3	ENLMQVYQQARL	TolC	Outer membrane protein TolC	54.0	5.34
4	ENLMQVYQQ	TolC	Outer membrane protein TolC	54.0	5.34
5	ATGKIVQVI	AtpB	ATP synthase β chain (EC.3.6.1.34)	50.2	4.76
6	VDFHGYARSG	LamB	Maltoporin precursor	49.9	4.66
7	EVRIVIDSGV	TolB	Outer membrane protein TolB	46.0	7.72
8	XXLNIKTMIIP	DacA	D-Alanyl-D-alanine carboxypeptidase fraction A (EC.3.4.16.4)	44.4	8.55
9	ATSTVTGGYA	OmpX	Outer membrane protein OmpX	18.6	7.11

Name, function and molecular weight (Mw) were obtained by homology analysis of the amino acid sequences, and the isoelectric point (pI) was calculated from the amino acid composition of the predicted proteins.

the efflux pump against antimicrobial agents (TolB, TolC and maltoporin). However, to elucidate the QAC-resistant mechanism in detail, it would be necessary to confirm the general tendency of the protein analysis data among resistant strains and to study the functions of those proteins in the resistant cell and the relation between them and the resistance-acquiring process.

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