Effects of β-Lactamases and *omp* Mutation on Susceptibility to β-Lactam Antibiotics in *Escherichia coli*

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Four types of β-lactamases consisting of a penicillinase type I (TEM-1), a penicillinase type II (OXA-1), a cephalosporinase of *Citrobacter freundii*, and a cephalosporinase of *Proteus vulgaris* were introduced into *Escherichia coli* MC4100 and its *omp* mutants, MH1160 (MC4100 *ompR1*) and MH760 (MC4100 *ompR2*), by transformation. Effects of the combination of the *omp* mutations and these β-lactamases on the susceptibility of *E. coli* strains were studied with 15 β-lactam antibiotics including cephalosporins, cephamycins, penicillins, imipenem, and aztreonam. The *ompR1* mutant, MH1160, lacks OmpF and OmpC, and it showed reduced susceptibility to 11 of the 15 β-lactam agents. The reduction in susceptibility to cefoxitin, moxalactam, and flomoxef was much greater than reduction in susceptibility to the other agents. When the *ompR1* mutant produced the cephalosporinase of *C. freundii*, the susceptibility of the mutant to 12 of the 15 β-lactam antibiotics decreased. The reduction in susceptibility of MH1160 to 10 of the 12 agents affected by the enzyme was two- to fourfold greater than that observed in MC4100. Such a synergistic effect was also observed with the cephalosporinase of *P. vulgaris* and *ompR1* mutation against six cephalosporins, moxalactam, and aztreonam.

β-Lactamases of gram-negative bacteria play an important part in bacterial resistance to β-lactam antibiotics. They are mediated by chromosomes or by plasmids. Both expression of β-lactamases, i.e., amount of the enzymes, and substrate profiles of the enzymes are major factors that determine resistance levels. Also, permeability of β-lactam antibiotics through the bacterial outer membrane affects susceptibility to β-lactam agents (21). Since OmpF and OmpC porins of *Escherichia coli* were found to act as pores for diffusion through the outer membrane, the role of the porin channels in permeation of β-lactams has been studied actively (24, 29). Some β-lactam antibiotics, e.g., cefoxitin, are known to penetrate much more rapidly through the OmpF porin than through the OmpC porin (11). Decreased permeability of the outer membrane and alterations in the outer membrane proteins also have been studied as factors in resistance to β-lactam antibiotics (1, 6, 12). Recently, the contributions of β-lactamases and outer membrane permeability to resistance have been studied simultaneously (15, 20, 27). Such an approach is more appropriate when both hydrolytic rate and permeation rate are slow (5). In our studies on bacterial resistance to newer cephalosporins by cephalosporinases, a decrease in permeability intensified the reduction in antimicrobial activity by cephalosporinase against cefotaxime and ceftazidime in *E. coli* (7, 8). For the elucidation of the resistance mechanisms and the evaluation of new agents, studies on the effects of β-lactamase production on bacterial susceptibility of the mutants with altered outer membrane permeability should yield important information.

In this study, we chose four β-lactamases with different substrate profiles, two penicillinases and two cephalosporinases, and we also chose as substrates 15 β-lactam antibiotics including eight cephalosporins, two penicillins, two ox-

acephamycins, a cephamycin, a carbapenem, and a monobactam. The effect of β-lactamase production on susceptibility to β-lactam antibiotics was tested in *E. coli omp* mutants that lack OmpC or both OmpF and OmpC.

MATERIALS AND METHODS

Bacteria and plasmids. *E. coli* MC4100 (F⁻ ΔlacU169 araD139 rpsL relA thiA fibB), MH1160 (MC4100 *ompR1*), and MH760 (MC4100 *ompR2*) were kindly donated by S. Mizushima (Nagoya University, Nagoya, Japan). MH1160 and MH760 are *omp* mutants of MC4100. MH1160 lacks outer membrane proteins OmpF and OmpC (4). MH760 lacks OmpC and produces OmpF constitutively, while the parent strain MC4100 has osmoregulated production of OmpF and OmpC (3). Four plasmids, pMS510 and pMS509 (R. Okamoto, S. Mitsuhashi, and M. Inoue, unpublished results), pMS185-2, and pMS182-5, are derivatives of pACYC184 (2) and contain cloned β-lactamase genes of the following types: penicillinase type I (TEM-1) of Rms212 (28), penicillinase type II (OXA-1) of Rms213 (28), cephalosporinase of *Citrobacter freundii* GN346 (26), oxyimino-cephalosporinase of *Proteus vulgaris* GN7919 (16), respectively. pMS510 has an *Eco*RI fragment (14.5 kilobases [kb]) of Rms212 in the *Eco*RI site of pACYC184. pMS509 has an *Eco*RI fragment (7.4 kb) of Rms213 in the *Eco*RI site of pACYC184. The chromosomal cephalosporinase gene of *C. freundii* GN346 was cloned into the *Eco*RI site of pACYC184 to produce pMS185, which mediates inducible production of *C. freundii* cephalosporinase. pMS185-2 is a deletion derivative of pMS185 obtained by the use of *Acc*I and contains a 4.8-kb fragment from the *C. freundii* chromosome. First, the chromosomal cephalosporinase gene of *P. vulgaris* GN7919 was cloned into the *Eco*RI site of pACYC184 to yield pMS182 (14), which mediates inducible production of *P. vulgaris* cephalosporinase. Second, the cephalosporinase gene of MS182 was recloned into the same vector by the use of *Hae*II to produce pMS182-5, which contains a 6.35-kb fragment from the *P. vulgaris* chromosome. Transformants

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TABLE 1. β-Lactamase activity of *E. coli* strains with cloned β-lactamase genes

Strain[a]	Plasmid	Enzyme[b]	Enzyme activity (nkat/mg of protein)[c]	MIC (μg/ml)[d] ABPC	MIC (μg/ml)[d] CET
MC4100 (F⁺ C⁺)	Host	None	—	1.56	3.13
	pMS510	PCase type I	55	1,600	6.25
	pMS509	PCase type II	13	6,400	6.25
	pMS185-2	CSase	0.82	6.25	50
	pMS182-5	CXase	3.4	1,600	1,600
MH1160 (F⁻ C⁻)	Host	None	—	3.13	25
	pMS510	PCase type I	28	3,200	100
	pMS509	PCase type II	5.1	6,400	100
	pMS185-2	CSase	0.88	25	800
	pMS182-5	CXase	3.6	3,200	6,400
MH760 (F⁺⁺ C⁻)	Host	None	—	1.56	3.13
	pMS510	PCase type I	28	1,600	6.25
	pMS509	PCase type II	5.5	3,200	6.25
	pMS185-2	CSase	1.1	6.25	50
	pMS182-5	CXase	3.3	1,600	800

[a] F⁻ and C⁻, Lack of OmpF and OmpC, respectively; F⁺ and C⁺, osmoregulated production of OmpF and OmpC, respectively; F⁺⁺, constitutive production of OmpF.

[b] PCase, Penicillinase; CSase, cephalosporinase of *C. freundii*; CXase, cephalosporinase of *P. vulgaris*.

[c] —, Less than 0.08 nkat/mg for ampicillin and less than 0.05 nkat/mg for cephalothin.

[d] ABPC, Ampicillin; CET, cephalothin.

with pMS185-2 or pMS182-5 produced cephalosporinases constitutively. These plasmids were introduced into *E. coli* MC4100, MH1160, and MH760 by transformation. The transformants of MC4100 and MH1160 with pMS185-2 or pMS182-5 were reported previously (7).

Antibiotics. Antibiotics were obtained from the following companies: cefoxitin and imipenem, Banyu Pharmaceutical Co., Ltd.; cefepime (BMY-28142), Bristol-Myers Research Institute; ceftizoxime, Fujisawa Pharmaceutical Co., Ltd.; cefotaxime and cefpirome (HR810), Hoechst Japan Ltd.; ampicillin, Meiji Seika Kaisha Ltd.; cefuroxime and ceftazidime, Shinnihon Jitsugyo Co., Ltd.; cephalothin, moxalactam, and flomoxef, Shionogi & Co., Ltd.; aztreonam, Squibb Japan Inc.; and piperacillin and cefoperazone, Toyama Chemical Co., Ltd.

β-Lactamase assay. The β-lactamase activities of *E. coli* strains were determined by a spectrophotometric method (18) at 30°C in 50 mM phosphate buffer (pH 7.0) with ampicillin (100 μM) as the substrate for penicillinases and cephalothin (100 μM) as the substrate for the cephalosporinases. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per second (nanokatals) by 1 mg of protein of bacterial crude extract prepared by sonication. Protein concentration was determined by the method of Lowry et al. (13) with bovine serum albumin as the standard.

SDS-urea-PAGE. Outer membrane proteins of the strains of *E. coli* before and after transformation were confirmed by sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis (SDS-urea-PAGE) (19). The outer membrane fraction was prepared by the method of Inokuchi et al. (9) as a Sarkosyl-insoluble fraction. Penassay broth (Difco Laboratories) was used for a bacterial culture medium.

Susceptibility testing. Susceptibilities of the *E. coli* strains to various β-lactam antibiotics were determined by an agar dilution method in sensitivity test agar (Nissui Seiyaku), i.e., modified Mueller-Hinton agar. Approximately 10⁴ CFU of bacterial culture was inoculated onto agar plates containing serial twofold dilutions of the agents. MIC was scored after incubation for 18 h at 37°C. When a difference in MICs of an agent between two strains was fourfold or greater, the difference was considered significant.

RESULTS

β-Lactamase activities of the transformants. Table 1 shows β-lactamase activities of *E. coli* MC4100, MH1160, and MH760 and their transformants and MICs of ampicillin and cephalothin for the strains. The expression of the cephalosporinases mediated by pMS185-2 or pMS182-5 was almost the same among the transformants of MC4100, MH1160, and MH760, whereas the penicillinase activities, which were mediated by pMS510 and pMS509, of MH1160 and MH760 were about half of those of the transformants of MC4100. Susceptibility of the *E. coli* strains to ampicillin or cephalothin decreased significantly after transformation.

SDS-urea-PAGE of outer membrane proteins. The outer membrane proteins of the transformants were compared with those of the host strains. Figure 1 shows SDS-urea-PAGE patterns of the outer membrane proteins from the strains before and after transformation with pMS185-2 or pMS182-5. The strains containing pMS510 or pMS509 gave results comparable to those of the strains containing pMS185-2 or pMS182-5. MC4100 produced both OmpF and OmpC. MH1160 did not produce either OmpF or OmpC. MH760 did not produce OmpC and produced much more OmpF than did MC4100. The outer membrane proteins of the strains did not change after transformation.

Susceptibilities of the *E. coli* strains with and without β-lactamases to various β-lactam antibiotics. Susceptibilities of *E. coli* MC4100, its *omp* mutants, and their transformants with β-lactamases were tested against the following 13 β-lactam antibiotics in addition to ampicillin and cephalothin (Table 2; see Table 1 for susceptibilities to ampicillin and cephalothin): seven cephalosporins, i.e., cefoperazone, ceftizoxime, cefuroxime, cefotaxime, ceftazidime, cefpirome, and cefepime; a penicillin, piperacillin; two oxacephamycins, moxalactam and flomoxef; a cephamycin, cefoxitin; a carbapenem, imipenem; and a monobactam, aztreonam.
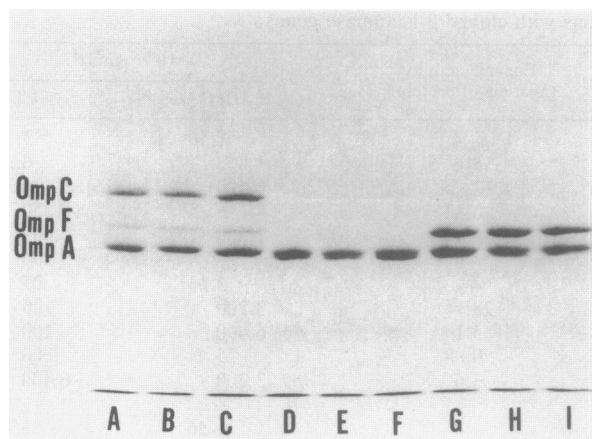
FIG. 1. SDS-urea-PAGE of the outer membrane proteins of *E. coli* strains before and after transformation with pMS185-2 and pMS182-5. Lanes: A, outer membrane proteins from MC4100; B, MC4100/pMS185-2; C, MC4100/pMS182-5; D, MH1160; E, MH1160/pMS185-2; F, MH1160/pMS182-5; G, MH760; H, MH760/pMS185-2; I, MH760/pMS182-5.

(i) Decrease in susceptibility by *ompR1* mutation. A decrease in susceptibility by *ompR1* mutation was observed with 11 of the 15 β-lactams tested but not with ampicillin, piperacillin, or imipenem. The activities of cefoxitin, moxalactam, and flomoxef were most affected by the mutation, and their MICs for MH1160 were 16-fold those for MC4100.

(ii) Decrease in susceptibility of MC4100 by production of β-lactamases. The type I penicillinase significantly reduced the activities of ampicillin, piperacillin, and cefoperazone but reduced only slightly or not at all the activities of the other β-lactam antibiotics. The type II penicillinase greatly reduced the activity of the penicillins, resulting in a more than 500-fold increase in MIC, and also reduced the activity of six cephalosporins, i.e., cefepime and cefpirome (128-fold

increase in MIC), ceftizoxime (32-fold increase in MIC), cefoperazone (16-fold increase in MIC), cefotaxime (16-fold increase in MIC), and cefuroxime (8-fold increase in MIC). The cephalosporinase of *C. freundii* reduced the activity of 10 of the 15 β-lactam agents. The increase in MIC was 16-fold for cephalothin and ceftizoxime; 8-fold for cefoxitin, flomoxef, and cefotaxime; and 4-fold for ampicillin, piperacillin, cefoperazone, ceftazidime, and aztreonam. Cefepime, cefpirome, cefuroxime, moxalactam, and imipenem were slightly or not affected by the enzyme in MC4100. The cephalosporinase of *P. vulgaris* reduced the activity of 11 of the 15 β-lactam agents. This enzyme affected aztreonam and all cephalosporins and penicillins tested. Among these cephalosporins, reduction in activity by the enzyme was smaller for ceftazidime, cefepime, and ceftizoxime than for the other seven cephalosporins; ceftazidime, cefepime, and ceftizoxime showed an increase in MIC of less than or equal to 16-fold. The reduction in antimicrobial activity was not significant for cefoxitin, moxalactam, flomoxef, or imipenem.

(iii) Combined effects of β-lactamase production and *omp* mutation. The greater increase in MIC for MH1160 than for MC4100 was considered due to a synergistic effect of *ompR1* mutation with β-lactamases. Synergistic effect of the *C. freundii* cephalosporinase and *ompR1* mutation was observed for 10 of the 12 β-lactams affected by the enzyme. A similar effect was observed with the *P. vulgaris* enzyme against 8 of 12 agents. Against ampicillin and piperacillin, type I and type II penicillinases and the *P. vulgaris* cephalosporinase did not show such a synergistic effect. Although the penicillinases affected antimicrobial activity synergistically with *ompR1* mutation against three of four cephalosporins (by type I penicillinase) and three of seven cephalosporins (by type II penicillinase), the synergistic effect was less distinct than those observed with the two cephalosporinases. The combined effects of β-lactamases and *omp* mutation on bacterial susceptibility were more significant when they

TABLE 2. Susceptibility of *E. coli omp* mutants and their β-lactamase-producing transformants to β-lactam antibiotics

Strain[a]	Plasmid	β-Lacta-mase[b]	MIC (μg/ml)[c]												
			PIPC	CFX	LMOX	FMOX	IPM	AZT	CXM	CPZ	CZX	CTX	CAZ	CPRM	CFPM
MC4100	Host	None	0.20	1.56	0.05	0.025	0.20	0.05	1.56	0.05	0.025	0.025	0.10	0.013	0.013
(F⁺ C⁺)	pMS510	PCase I	100	1.56	0.05	0.025	0.20	0.10	1.56	1.56	0.025	0.025	0.10	0.025	0.025
	pMS509	PCase II	100	1.56	0.05	0.025	0.20	0.10	12.5	0.78	0.78	0.39	0.10	1.56	1.56
	pMS185-2	CSase	0.78	12.5	0.10	0.39	0.20	0.20	3.13	0.20	0.39	0.20	0.39	0.025	0.013
	pMS182-5	CXase	100	1.56	0.10	0.025	0.20	12.5	>200	12.5	0.10	3.13	1.56	0.78	0.39
MH1160	Host	None	0.39	25	0.78	0.39	0.20	0.20	6.25	0.20	0.10	0.10	0.20	0.10	0.05
(F⁻ C⁻)	pMS510	PCase I	100	25**	0.78**	0.39**	0.20	0.20	6.25*	6.25*	0.10*	0.10*	0.20	0.39**	0.20**
	pMS509	PCase II	200	25**	0.78**	0.39**	0.20	0.20	50*	3.13*	6.25**	3.13*	0.20	12.5**	6.25**
	pMS185-2	CSase	1.56	200**	1.56**	12.5**	0.20	1.56**	12.5*	1.56**	6.25**	3.13**	3.13**	0.39**	0.20**
	pMS182-5	CXase	100	25**	3.13**	0.39**	0.39	100**	>200	200**	0.78**	25**	6.25**	12.5**	3.13**
MH760	Host	None	0.39	1.56	0.05	0.05	0.39	0.013	1.56	0.025	0.013	0.025	0.05	0.013	0.013
(F⁺⁺ C⁻)	pMS510	PCase I	100	1.56	0.05	0.05	0.39	0.013	1.56	0.39	0.013	0.025	0.05	0.025	0.013
	pMS509	PCase II	200	1.56	0.05	0.05	0.39	0.025	12.5	0.20	0.78	0.39	0.05	0.78	0.39
	pMS185-2	CSase	0.78	12.5	0.05	0.20	0.39	0.05	1.56	0.05	0.39	0.20	0.20	0.013	0.013
	pMS182-5	CXase	100	1.56	0.10	0.05	0.78	6.25	>200	12.5	0.10	3.13	0.78	0.39	0.20

[a] F⁻ and C⁻, Lack of OmpF and OmpC, respectively; F⁺ and C⁺, osmoregulated production of OmpF and OmpC, respectively; F⁺⁺, constitutive production of OmpF.

[b] PCase I, Penicillinase type I (TEM-1) of Rms212; PCase II, penicillinase type II (OXA-1) of Rms213; CSase, cephalosporinase of *C. freundii* GN346; CXase, cephalosporinase of *P. vulgaris* GN7919.

[c] Asterisks indicate fourfold (*) and greater than fourfold (**) increases in MICs for MH1160 containing these plasmids over MICs for MC4100 containing corresponding plasmids. AZT, aztreonam; CAZ, ceftazidime; CFPM, cefepime; CFX, cefoxitin; CPRM, cefpirome; CPZ, cefoperazone; CTX, cefotaxime; CXM, cefuroxime; CZX, ceftizoxime; FMOX, flomoxef; LMOX, moxalactam; IPM, imipenem; PIPC, piperacillin.

were compared between MH1160 and MH760. MH760 showed a susceptibility to β-lactam antibiotics equal to or somewhat higher than that of MC4100, and its transformants were more susceptible to some of the β-lactam antibiotics than the transformants of MC4100.

DISCUSSION

The OmpF- and OmpC-deficient mutant of *E. coli*, MC4100 *ompR1*, showed reduced susceptibility to certain β-lactam antibiotics, including cephalosporins, oxacephamycins, cefoxitin, and aztreonam. The reduction of susceptibility was much more extensive for cefoxitin and oxacephamycins than for other β-lactam antibiotics. Cephalosporins showed relatively less increase in MIC after the mutation than did the cephamycins. These observations agree with the results of other investigators (11, 12). The lower susceptibility of OmpF- and OmpC-deficient mutants has been considered due to the lower permeability of β-lactam antibiotics through their outer membranes (6). Although the increase in MIC of the cephalosporins and aztreonam was small, i.e., an eightfold or smaller increase, it augmented the reduction in antimicrobial activity by β-lactamases: the increase in MIC by the cephalosporinases of *C. freundii* and *P. vulgaris* was two- to fourfold greater than the additive effects of the *ompR1* mutation and the β-lactamases. The enhancement of resistance due to β-lactamases in MH1160(OmpF⁻ OmpC⁻) was significant against cephems and aztreonam but not against penicillins and imipenem. These facts indicate a much greater dependency of cephems and aztreonam than penicillins and imipenem on the OmpF and OmpC porins in penetration through the outer membrane. Such synergistic effects were less significant with the two penicillinases, type I (TEM-1) and type II (OXA-1), probably because the expression of the penicillinases in the *omp* mutant was about half that in the parent strain, for unknown reasons. The lack of OmpC alone apparently did not result in enhancement of β-lactamase-mediated resistance in MH760 (OmpC⁻ OmpF⁺⁺), which still produces OmpF constitutively. However, OmpC⁻ mutants with osmoregulated OmpF would behave as OmpF⁻ and OmpC⁻ mutants under high-osmolality circumstances (17).

A recent problem in β-lactam chemotherapy is the existence or the emergence of multiple-β-lactam-resistant strains of gram-negative bacteria, e.g., *Enterobacter cloacae*, *C. freundii*, *Serratia marcescens*, and *Pseudomonas aeruginosa*, which overproduce chromosomal cephalosporinases (22). When the *ompR1* mutant produced the cephalosporinase of *C. freundii*, it showed decreased susceptibility to almost all β-lactam antibiotics, except for imipenem, compared with the parent. Among these β-lactam agents, cefepime and cefpirome showed much greater activity than other agents. This greater activity is mainly due to their much greater stability against the cephalosporinases at a low concentration which is still enough to inhibit penicillin-binding proteins, because of the low affinity of the enzymes for both agents (8, 23). Neither production of these β-lactamases nor *ompR1* mutation significantly affected the activity of imipenem. These observations indicate high permeability of imipenem through the outer membrane (7) and a possibility that imipenem penetrates into periplasmic space through an alternative pathway, as discussed by Jaffe et al. (11). Our results confirm the observation of Jacoby and Sutton (10) that some TEM-type and OXA-type enzymes and *E. coli* cephalosporinase affect the activity of newer broad-spectrum β-lactam antibiotics such as cefotaxime,

ceftazidime, cefepime, and aztreonam. They also studied effects of β-lactamases in *omp* mutant strains. However, our results revealed more clearly the synergistic effects of lowered permeability and β-lactamase production on bacterial susceptibility.

Substrate profiles of β-lactamases are major factors in resistance. In fact, the *P. vulgaris* cephalosporinase possesses a broad substrate profile, ranging from penicillins to cephalosporins including oxyimino-cephalosporins (16), and this cephalosporinase reduced antimicrobial activity of penicillins, cephalosporins, and aztreonam in *E. coli* (Table 2). Among the β-lactam antibiotics we tested, the low-permeability strain, MH1160, with the *P. vulgaris* enzyme was still susceptible to cefepime, ceftizoxime, flomoxef, imipenem, and moxalactam: the MICs were lower than 6.25 μg/ml. Since a derepressed and overproducing mutant of *P. vulgaris* was still susceptible to cefotaxime and ceftazidime (7), the chromosomal cephalosporinase of *P. vulgaris* has less clinically significant resistance to the newer-generation cephalosporins. Recently, however, a new type of plasmid-mediated β-lactamase with a broad substrate spectrum was reported, i.e., CTX-1 (25), which is similar to the *P. vulgaris* enzyme in hydrolysis of cefotaxime and inhibition by clavulanic acid. These broad-spectrum β-lactamases may become more important factors in the resistance to β-lactam antibiotics when pathogenic organisms have low permeability to β-lactam antibiotics. Our set of *E. coli* strains should be useful for evaluation of β-lactam antibiotics because it combines both a variety of β-lactamases and β-lactam permeability in isogenic strains.

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